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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

V. EXTRACELLULAR PEPTIDASES PRODUCED BY FUNGI GROWN IN SUBMERGED CULTURE[1]

BY W. B. MCCONNELL,[2] E. Y. SPENCER,[3] AND J. A. TREW[4]

ABSTRACT

The culture media from selected fungi grown in submerged culture have been shown to contain enzymes capable of hydrolyzing some synthetic dipeptides and their derivatives. Readily measurable amounts of aminopeptidase, carboxypeptidase, and dipeptidase activity were found, and differences between the systems elaborated by different organisms were observed. The most rapid hydrolysis of N-cbzo-α-L-glutamyl-L-glutamic acid (I) and of N-cbzo-α-L-glutamyl-DL-alanine (II) by *Gliocladium roseum* PRL 86 occurred at pH 4.8 to 4.9. I was hydrolyzed most rapidly by *Alternaria tenuis* PRL 369 at pH 4.7. The activation energies for the hydrolysis of I by PRL 369 and of II by PRL 86 were found to be 11,000 and 15,000 calories per mole, respectively. The activation energy for the hydrolysis of I by PRL 86 was estimated as being between 10,000 and 13,000 calories per mole.

INTRODUCTION

Although the proteolytic systems elaborated by molds and actinomycetes have been known and studied for a number of years, there is little information available regarding the number and nature of the individual proteases present in the crude enzyme preparations. The work of Berger, Johnson, and Peterson (2) suggests that these are fairly complex mixtures. In 1936 they made a study of 30 common molds and reported that extracts of the mycelium contained a proteinase and at least five peptidases; a carboxypolypeptidase, an aminopolypeptidase, a dipeptidase, and two enzymes that hydrolyze diglycine and triglycine. Investigations of proteases excreted into the culture medium have been particularly limited. Crewther (12) reported the isolation of crystals containing proteinase from a medium in which *Aspergillus niger* had grown in still culture. Dion (13) studied the optimum conditions for production of extracellular proteases by a number of molds and actinomycetes grown in submerged culture. McConnell (19) found that crude preparations from these culture media were capable of hydrolyzing a number of proteins at a pH near 8, and that in general a considerable amount of free amino acid was liberated. It seems probable that

[1] *Manuscript received April 13, 1953.*

Contribution from the Prairie Regional Laboratory, National Research Laboratories, Saskatoon, Sask., and Department of Chemistry, University of Saskatchewan. Issued as Paper No. 154 on the Uses of Plant Products and as N.R.C. No. 3024. From a thesis presented to the School of Graduate Studies, University of Saskatchewan, by J. A. Trew in partial fulfillment of the requirements for the degree of Master of Arts.

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the extracellular proteases, like those obtained from mycelium (3), contain a number of different proteolytic constituents.

It appeared of interest to further characterize these crude mold enzymes by using synthetic substrates. The results indicate that the crude extracellular protease systems may be considered as complex systems. Some of the characteristics of the carboxypeptidase components are discussed.

EXPERIMENTAL

Preparation of Enzymes

Crude fungal proteases were produced by growing the organisms in submerged culture using a Klim–glucose medium containing either calcium or magnesium carbonate. They were supplied by Dr. W. M. Dion who has described their preparation in detail (13). At the end of the growth period the medium was freed of mycelium by filtration, lyophilized, and stored dry until required for hydrolysis studies. The organisms used were *Mortierella renispora* PRL 26, *Gliocladium roseum* PRL 79, *Gliocladium roseum* PRL 86, *Trichoderma viride* PRL 92, *Fusarium* sp. PRL 232, *Chaetomium* sp. PRL 319, *Alternaria tenuis* PRL 369, and *Streptomyces* sp. PRL 376. Throughout the remainder of this paper the enzymes are referred to by the identification number of the organisms from which they were produced.

Preparation of Substrates

A list of the substances used together with some comments on their preparation follows.

N-Cbzo-glyclglycine,* m.p. 178°C. (reported m.p. 178°C. (5)), was prepared according to the method of Hanson and Smith (16). Hydrogenolysis in acetic acid with platinum oxide catalyst yielded glycylglycine, decomposed 215–220°C. (reported m.p. 215–220°C. with decomposition (14)). The mixture of diastereoisomers, N-cbzo-α-L-glutamyl-DL-alanine ethyl ester, was prepared according to the procedure of Bergmann, Zervas, and Fruton (6,17) and the substance was hydrolyzed to N-cbzo-α-L-glutamyl-DL-alanine which was recrystallized from ethyl acetate to a composition melting at 153°C. The same method was used to prepare N-cbzo-α-L-glutamylglycine with a melting point of 141.5°C. (reported m.p. 143°C. (15)). N-Cbzo-α-L-glutamyl-L-glutamic acid diethyl ester, prepared as described by Bergmann (5,11), was saponified with *N* sodium hydroxide for 45 min. The mixture was acidified with hydrochloric acid and N-cbzo-α-L-glutamyl-L-glutamic acid was recovered as a precipitate. It sintered at 140–145°C., m.p. 174°C. (reported to sinter at 145°C., m.p. 176°C. (5)). Hydrogenolysis of 2.8 gm. of N-cbzo-α-L-glutamyl-L-glutamic acid diethyl ester in 25 ml. of absolute ethanol, 2 ml. of glacial acetic acid, and 0.1 gm. of platinum oxide yielded α-L-glutamyl-L-glutamic acid diethyl ester. The catalyst was removed by filtration and the solvent evaporated *in vacuo*. After recrystallization from ethanol it melted at 136–137°C. (reported m.p. 137°C. (5)). α-L-Glutamyl-L-glutamic acid was prepared by hydrogenolysis of N-cbzo-α-L-glutamyl-L-glutamic acid in glacial acetic acid. The residue was taken up in a minimum

* *The term "carbobenzoxy" is abbreviated as "cbzo" throughout this report.*

volume of water, two or three volumes of ethanol were added, and after it had stood for two days in the cold the precipitate was collected. M.p. 190°C. (reported m.p. 205°C. (5), 184–185°C. (9)).

Methods for Hydrolysis Study

Estimation of peptidase activity was done by observing the rate of liberation of free amino acids from buffered solutions of the synthetic substrates under controlled conditions of temperature. Digestion mixtures contained 1.0 ml. of substrate solution (0.125 *M*), 0.50 ml. of buffer solution (1 *M*), and 0.75 ml. of enzyme solution (dried culture medium diluted to one-half original volume). Cobalt chloride (0.01 *M*, 0.25 ml.) was also added to give a total volume of 2.5 ml. The mixture was incubated in a water bath controlled at 37°C. ± 0.05°C. Aliquots of 0.20 ml. were removed immediately after mixing and at convenient time intervals thereafter. These were analyzed for free amino acids according to a ninhydrin procedure described by McConnell (20). Two drops of dilute sulphuric acid were added to the sample of digestion mixture in the microdiffusion cells to help lower the pH of the heavily buffered solutions to the required value. The increase in free amino acids during the digestion was expressed in terms of percentage hydrolysis of the peptide bond in the substrate taken. The increase in free amino acid during the digestion was assumed to arise from the hydrolysis of peptide bonds. Hydrolysis of a peptide bond in a dipeptide would yield two free amino acid molecules whereas hydrolysis of a monosubstituted dipeptide would yield only one amino acid.

EXPERIMENTAL RESULTS AND DISCUSSION

Demonstration of Peptidase Activity

Eight organisms previously shown to produce appreciable amounts of proteo-

TABLE I

DEMONSTRATION OF PEPTIDASE ACTIVITY IN EXTRACELLULAR FUNGAL PROTEASES

		PERCENT HYDROLYSIS							
		ENZYME							
SUBSTRATE	APPROX. pH	PRL 26	PRL 79	PRL 86	PRL 92	PRL 316	PRL 319	PRL 369	PRL 376
α-L-glutamyl-L-glutamic Acid	3.5	0	0	0	0	0	0	0	0
N-cbzo-α-L-glutamyl-L-glutamic Acid	5.5	9.6	76	97	45	3.0	40	51	1.0
N-cbzo-α-L-glutamyl-DL-alanine	7.5	16	23	40	9.6	18	0	0	7.6
α-L-glutamyl-L-glutamic Acid diethyl Ester	10.5	32	7.8	21	10	19	7.1	25	6.7
N-cbzo-α-L-glutamyl-glycine	11.0	33	18	2.1	10	28	28	24	10

lytic activity (13) were tested for their ability to hydrolyze a number of peptides and their derivatives. Activity toward the synthetic materials was fairly general. The observations are illustrated in Table I and are a selection of results obtained after digestion periods of 24 hr.

The data in Table I may be supplemented by noting that in 20 hr. a preparation of PRL 79 in phosphate buffer at pH 7.6 was found to hydrolyze 30% of the N-cbzo-glycylglycine and 4.3% of the glycylglycine. Low pH (3.5) probably accounts, in part, for the observed absence of activity toward α-L-glutamyl-L-glutamic acid. At least some of the preparations digested the latter slowly above pH 4, and PRL 26, PRL 86, and PRL 369 in faintly alkaline solution (pH 7.2–7.4) caused 5.6, 9.5, and 6.5% hydrolysis respectively in 24 hr. at 37°C.

Table I shows that each enzyme used was capable of digesting one or more of the substrates to the extent of at least 10% in 24 hr. Since the survey does not constitute a study of the many variable conditions, it is probable, that with the use of optimum conditions, much greater activity would be observed. The activity toward all of the substrates of the type N-cbzo-α-L-glutamyl-L-glutamic acid, α-L-glutamyl-L-glutamic acid diethyl ester, and α-L-glutamyl-L-glutamic acid indicates the presence of carboxypeptidase, aminopeptidase, and dipeptidase (3). Berger, Johnson, and Peterson (2) had observed similar peptidases in mycelium extracts, and it is therefore not surprising that they also occur as extracellular proteases.

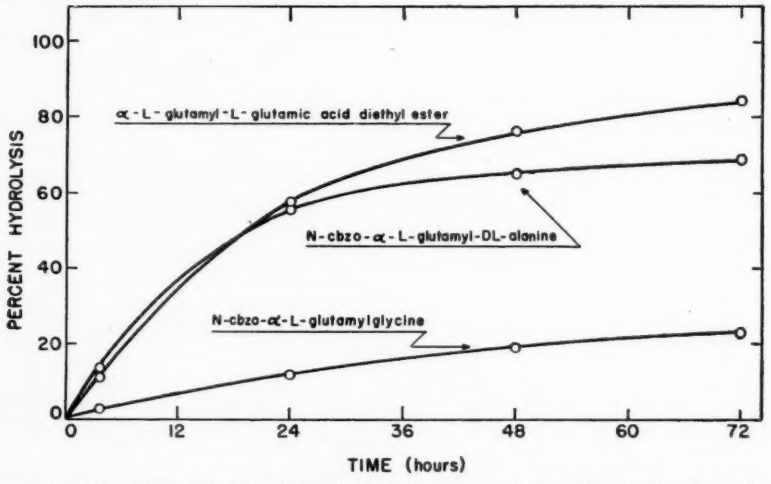

FIG. 1. The effect of time on the hydrolysis of some synthetic substrates by PRL 86.

Fig. 1 is typical of results obtained when the progress of the digestion was followed for several days. The hydrolyses were done at 37°C. in phosphate buffer pH 6. The N-cbzo-α-L-glutamylglycine was hydrolyzed very slowly at this pH; only about 23.8% of it being consumed during a 72 hr. period. In the early stages of digestion the rates of degradation of N-cbzo-α-L-glutamyl-DL-alanine and of α-L-glutamyl-L-glutamic acid diethyl ester were practically the same. After 50 to 60% of these materials had been hydrolyzed it became

apparent that the rate of liberation of amino acids from the alanine-containing peptides was decreasing rapidly. At the end of 72 hr. only 69.5% of N-cbzo-α-L-glutamyl-DL-alanine had reacted, whereas over 85% of the α-L-glutamyl-L-glutamic acid diethyl ester had been hydrolyzed. Although a number of alternative explanations are possible it is interesting to note that the rapid decrease in hydrolysis rate of the diastereoisomeric mixture is in harmony with previous observations that optical isomers react at different rates in the presence of enzyme catalysts (6, 7). The observed hydrolysis of 69.5% for the peptide derivative of DL-alanine nevertheless shows that at least some of both isomers was being hydrolyzed.

Studies with N-Substituted Dipeptides

The experiments recorded so far have suggested that the extracellular peptidase systems of the molds used are of considerable complexity. It is evident that to understand the effects of variables such as pH and temperature, separate consideration of each enzyme–substrate system is required. Attention was therefore directed toward a somewhat more detailed study of the action of PRL 86 and PRL 369 on some N-substituted dipeptides.

The results of measurement of the effect of pH on three such enzyme–substrate combinations are given in Fig. 2. The digestions were carried out for

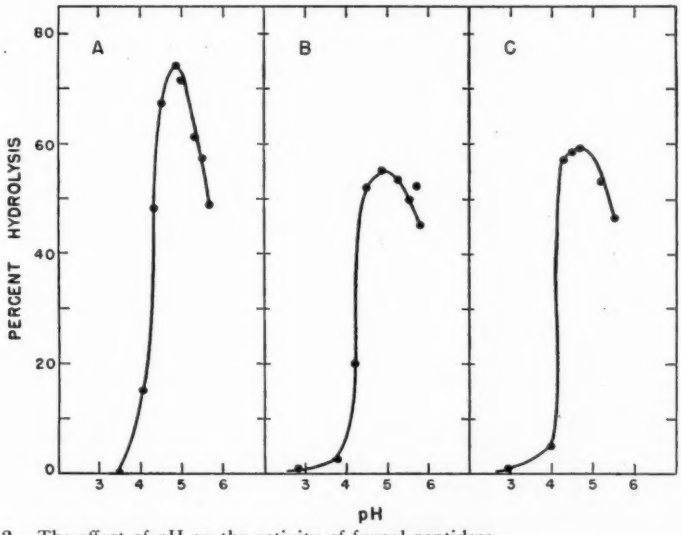

FIG. 2. The effect of pH on the activity of fungal peptidase.
A. N-Cbzo-α-L-glutamyl-L-glutamic acid digested with PRL 86.
B. N-Cbzo-α-L-glutamyl-DL-alanine digested with PRL 86.
C. N-Cbzo-α-L-glutamyl-DL-alanine digested with PRL 369.

24 hr. in phthalate buffer using methods outlined. The evidence of a fairly sharp optimum pH at pH 4.8 to 4.9 is of particular interest. Previous reports of peptidase activity would suggest maximum activity nearer neutrality (18, 21, 4). It is also of interest to note that the proteases were previously reported to have maximum activity above pH 7 (19, 23). The observed low activity at neutral or

slightly alkaline pH would appear to be characteristic of the carboxypeptidase component and of the particular substrates used rather than being a general property of the fungal protease systems.

Some experiments on pH effect with a phosphate buffer showed that the nature of the buffer itself had an influence on activity. The per cent hydrolysis in 24 hr. obtained with PRL 86 acting on N-cbzo-α-L-glutamyl-L-glutamic acid and on N-cbzo-α-L-glutamyl-DL-alanine was only about three quarters as much with the phthalate buffer as when a phosphate buffer was used at the same pH. As only a very narrow pH range was common to both these buffers, good quantitative comparison was difficult. The shape of the curve with phosphate buffers at different pH values was, so far as could be observed, the same as with the phthalate buffer and suggests maximum digestion rates near or below pH 5.

The hydrolysis rates of some enzyme–substrate combinations were followed for about 14 hr. at three different temperatures. Fig. 3 contains the results for N-cbzo-α-L-glutamyl-DL-alanine when hydrolyzed with PRL 86. Since the lines are approximately linear during the first four or five hours, the slopes were used to characterize the digestion rates at each temperature. The slopes of the lines at 36.5°C., 25°C., and 14.5°C. were estimated from the curves of Fig. 3 as being 4.35, 2.20, and 1.1% hydrolysis per hour.

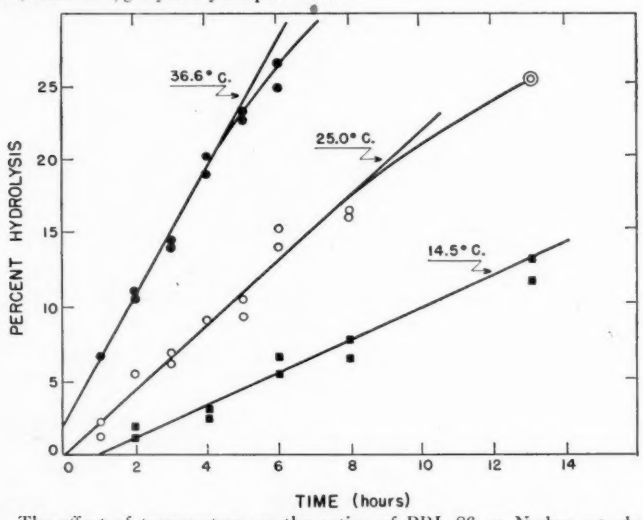

FIG. 3. The effect of temperature on the action of PRL 86 on N-cbzo-α-L-glutamyl-DL-alanine.

A linear relation was observed between the reciprocal of the absolute temperature and the logarithm of the slopes. The activation energy as estimated from these results by conventional methods would be about 11,000 calories per mole. Similar curves were obtained using PRL 369 to digest N-cbzo-α-L-glutamyl-L-glutamic acid, at the three temperatures. An activation energy near 15,000 cal. was found. Results with PRL 86 and N-cbzo-α-L-glutamyl-L-glutamic acid were more erratic and by the slopes of the lines an Arrhenius plot with some deviation

from linearity was noted. It was estimated however that the activation energy for the temperature range used was somewhere between 10,000 cal. and 13,000 cal. per mole. The activation energies obtained in this work are of the same order as other values previously reported for the enzymatic hydrolysis of proteins and synthetic protease substrates. Casein at pH 8.1 and benzoyl-L-arginine amide at pH 7.8 when hydrolyzed with trypsin have been reported to give activation energies of 15,400 (22) and 14,900 (10) respectively. Similarly chymotrypsin hydrolysis of casein at pH 7.7 and of benzoyltyrosylglycyl amide at pH 7.7 gave activation energies of 12,000 (1) and 10,500 (10) respectively.

Attempts were made to compare the crude mold proteases with pancreatin (Takamine Laboratory, Clinton, N.S.) and with pepsin (Nutritional Biochemicals, Cleveland, Ohio) with regard to rate of hydrolysis of the synthetic substrates. Although the mold enzymes were found to attack the synthetic substrates, no digestion by pepsin of N-cbzo-α-L-glutamyl-L-glutamic acid, α-L-glutamyl-L-glutamic acid diethyl ester, or N-cbzo-α-L-glutamyl-DL-alanine was observed either at pH 2.5 or 4.6. The pepsin solution used for addition to the digestion mixture contained 20 mgm. per ml. Results with the same concentration of pancreatin were not reproducible, probably because considerable amounts of free amino acids were found to be liberated from the pancreatin preparation itself. Although quantitative data are not reported for pancreatin, it appeared to have no greater activity toward these substrates than the mold enzyme preparations. The following observations obtained with the substrate poly-γ-methyl-L-glutamate* are of interest. PRL 369 at pH 5.7 liberated about 7.8% of the glutamic acid from a 0.57% dispersion of the polymer in 20 hr. PRL 86 at pH 5.7 and pepsin at pH 3 liberated no measurable amount of glutamic acid and little or no hydrolysis occurred with pancreatin at pH 8.5. This is in agreement with the results of Blakley *et al.* (8) who found in addition slight hydrolysis of a copolymer of polylysine and glutamic acid and greater hydrolysis with the more complex copolymer of polylysine – glutamic acid.

No evidence was obtained to indicate that cobalt was necessary as an activator for the mold peptidases. Its use in all the digestion mixtures was only justified as a precaution to help ensure that maximum activity was obtained. Ions such as Zn^{++}, Mn^{++}, and Mg^{++} which are also known to take part in the action of metal peptidase were not added. The Klim-containing medium in which the enzymes were produced should supply requirements for these ions.

Much additional work is required to develop a more complete picture of the nature of the extracellular proteolytic systems produced by the molds. The present work suggests that they are complex mixtures of enzymes. The experiments on the effect of pH, although suggesting a rather unique and characteristic pH optimum toward the substrates available, fail to distinguish between the carboxypeptidases from the different molds and should be repeated on a variety of different proteases. Similarly the results from the study on temperature effects tend to suggest a similarity rather than differences between the extra-

* *Supplied by Mr. L. Wiseblatt, University of Saskatchewan. Average chain length: about eight glutamic acid residues.*

cellular carboxypeptidases of the molds used. The information obtained through this work should also prove useful in attempts to fractionate different types of proteolytic activity in the crude preparations. It is probable that further work would be most profitable if done with preparations which had been at least partially purified.

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THE POTENTIOMETRIC TITRATION OF CARBONATE SOLUTIONS CONTAINING URANIUM[1]

By J. Halpern

ABSTRACT

Potentiometric titrations with hydrochloric acid were carried out on standard sodium carbonate solutions containing varying amounts of uranyl nitrate. The results confirmed the fact that uranium is present in such solutions as the complex ion $UO_2(CO_3)_3^{\equiv}$. It was found that only the free CO_3^{--} is titrated with H^+ up to the first end point at pH = 8.2. The complex ion is very stable and is decomposed only on further addition of acid when the complexed CO_3^{--} along with the HCO_3^- in the solution is converted to carbonic acid before the second end point. The pH at which the second end point occurs is lowered from its normal value of 4.0 in the presence of uranium. This effect is attributed to hydrolysis of the UO_2^{++} ion. The necessary corrections for determining carbonate and bicarbonate in the presence of uranium are given.

INTRODUCTION

Carbonate solutions are commonly used to extract uranium from its ores (2, 3). The determination of the CO_3^{--} and HCO_3^- concentrations in the leach solutions containing uranium, by the normal procedure (5) of titrating with standard hydrochloric acid to consecutive end points at pH values of 8.2 and 4.0, is complicated by the formation of a complex ion (6) between UO_2^{++} and CO_3^{--} as follows:

$$UO_2^{++} + 3\,CO_3^{--} \rightarrow UO_2\,(CO_3)_3^{\equiv}. \qquad [1]$$

The interpretation of the titration results depends on whether the CO_3^{--} tied up in this complex is converted to HCO_3^-, along with the free CO_3^{--}, before the first end point, or whether the complex is sufficiently stable to prevent this reaction from occurring until a lower pH is reached.

To obtain quantitative information on this point, a study was made in which titration curves were obtained for a series of solutions containing known amounts of UO_2^{++} and CO_3^{--}.

EXPERIMENTAL

Materials

Solutions were prepared by dissolving weighed amounts of reagent grade $UO_2(NO_3)_2.6H_2O$ and sodium carbonate in distilled water.

Titration Procedure

Aliquots of solutions of known composition were titrated with standard hydrochloric acid and the pH change followed with a Beckman Model H2 pH meter. Titration curves were plotted and end points corresponding to maxima in the slopes of these curves were determined in the usual manner (4). All titrations were carried out at room temperature (20°C.).

[1] *Manuscript received February 4, 1953.*
Contribution from the Department of Mining and Metallurgy, University of British Columbia, Vancouver, B.C. This work was supported by funds provided by the Atomic Energy Control Board and administered by the National Research Council of Canada.

RESULTS

A series of five solutions containing 4.71×10^{-2} M./l. sodium carbonate and amounts of $UO_2(NO_3)_2$ ranging from zero to 7.13×10^{-3} M./l. were titrated with 0.0984 *N* hydrochloric acid. The titration curves are shown in Fig. 1. The volumes of hydrochloric acid corresponding to the first and second end points, for each solution, are given in Table I along with the pH values at which these end points occurred.

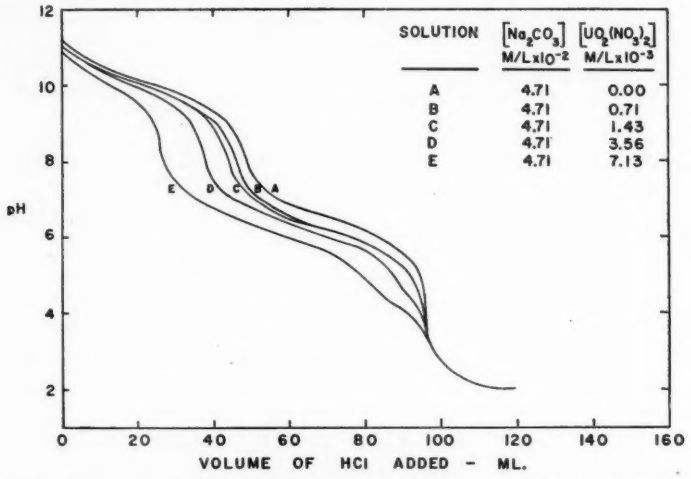

FIG. 1. Titration curves for sodium carbonate solutions containing varying amounts of $UO_2(NO_3)_2$. One hundred milliliter samples titrated with 0.0984 *N* hydrochloric acid.

TABLE I

TITRATION OF VARIOUS SOLUTIONS OF Na_2CO_3 AND $UO_2(NO_3)_2$ WITH 0.0984 *N* HCl
VOLUME OF EACH SOLUTION TITRATED (*V*)—100 ML.

Solution	Total concentrations (M./l.)			First end point			Second end point		
	$[CO_3^{--}]T$	$[HCO_3^-]$	$[UO_2^{++}]$	Vol. HCl(V_1), ml.	ΔV_1, ml.	pH	Vol. HCl(V_2), ml.	ΔV_2, ml.	pH
A	4.71×10^{-2}	0.0	0.0	48.0	0	8.25	96.0	0	4.0
B	"	"	7.13×10^{-4}	46.0	−2.0	8.1	96.0	0	3.8
C	"	"	1.43×10^{-3}	43.5	−4.5	8.2	96.0	0	3.8
D	"	"	3.56×10^{-3}	37.5	−10.5	8.2	97.0	1.0	3.4
E	"	"	7.13×10^{-3}	26.5	−21.5	8.25	97.0	1.0	3.2

As the concentration of UO_2^{++} in the solution is progressively increased from zero to 7.13×10^{-3} M./l. the following effects are noted.

1. The volume of hydrochloric acid corresponding to the first end point (at pH = 8.2) is reduced from 48.0 ml. to 26.5 ml.

2. The total amount of hydrochloric acid corresponding to the second end point remains substantially unchanged at 96.5 ± 0.5 ml.

3. While the pH at which the first end point occurs remains substantially

unchanged at 8.2, the pH of the second end point is progressively lowered from 4.0 to 3.2.

DISCUSSION

These results confirm the fact that the uranium is present quantitatively in the solutions examined as the complex ion, $UO_2(CO_3)_3^{\equiv}$. The complex appears to be very stable and is not decomposed at the equivalence point for the conversion of free CO_3^{--} to HCO_3^-. For a solution containing a given total concentration of CO_3^{--}, the shift in the first end point, ΔV_1, which marks this equivalence point, is thus related to the concentration of uranium, $[UO_2^{++}]$, as follows:

$$-\Delta V_1 = 3V[UO_2^{++}]/N \qquad [2]$$

where V is the volume (ml.) of the sample being titrated, and N is the normality of the hydrochloric acid.

This is confirmed in Fig. 2 where the experimental points for $-\Delta V_1$, taken from Table I, are seen to fall within experimental accuracy on a plot of $3V[UO_2^{++}]/N$.

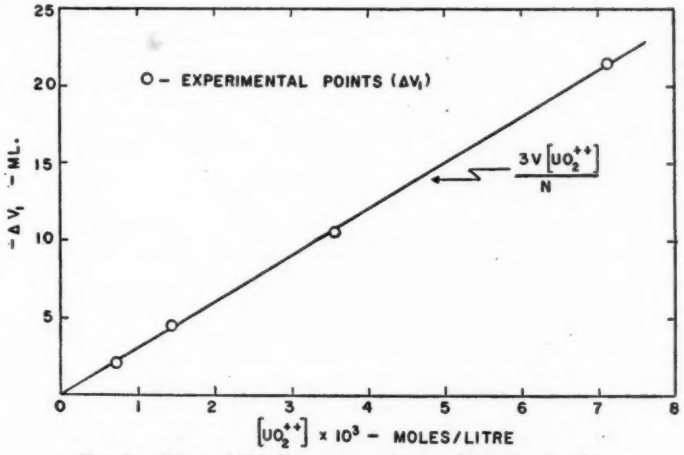

FIG. 2. Effect of UO_2^{++} concentration on the first end point.

The first end point in the titration, at pH 8.2, thus corresponds to the equivalence point of the reaction,

$$(CO_3^{--})_F + H^+ \rightarrow HCO_3^-. \qquad [3]$$

The titration results also demonstrate that the $UO_2(CO_3)_3^{\equiv}$ ion does break down before the second end point at pH 4 is reached and the CO_3^{--} liberated is converted to carbonic acid. The second end point thus corresponds to the equivalence points of the following reactions:

$$HCO_3^- + H^+ \rightarrow H_2CO_3, \qquad [4]$$

$$UO_2(CO_3)_3^{\equiv} + 6H^+ \rightarrow UO_2^{++} + 3\,H_2CO_3. \qquad [5]$$

Thus the total amount of hydrochloric acid required to give the second end point is independent of the UO_2^{++} concentration. This is also confirmed by the results in Table I.

The concentrations of CO_3^{--} and HCO_3^- in a solution containing uranium

are thus related to the first and second titration end points, V_1 and V_2, by the following equations:

$$[CO_3^{--}]_F = (V_1/V) \times N, \quad [6]$$

$$[CO_3^{--}]_T = (V_1/V) \times N + 3\,[UO_2^{++}], \quad [7]$$

$$[HCO_3^-] = [(V_2 - 2V_1)/V] \times N - 6\,[UO_2^{++}], \quad [8]$$

where $[CO_3^{--}]_F$ and $[CO_3^{--}]_T$ are the concentrations of free and total CO_3^{--} respectively.

The corrected values of $[CO_3^{--}]_F$, $[CO_3^{--}]_T$, and $[HCO_3^-]$, calculated from the titration results using these relations, are given in Table II and are seen to agree within experimental accuracy with the known values.

TABLE II

CALCULATION OF $[CO_3^{--}]$ AND $[HCO_3^-]$ FROM TITRATION RESULTS

Solution	$[UO_2^{++}]$, M./l.	True concentration (M./l.)			Apparent conc. (M./l.)	Corrected concentration (M./l.)		
		$[CO_3^{--}]_T$	$[CO_3^{--}]_F$*	$[HCO_3^-]$	$[HCO_3^-]$	$[CO_3^{--}]_F$	$[CO_3^{--}]_T$	$[HCO_3^-]$
A	0.0	4.71×10^{-2}	4.71×10^{-2}	0.0	0.0	4.71×10^{-2}	4.71×10^{-2}	0
B	7.13×10^{-4}	"	4.50×10^{-2}	"	0.36×10^{-2}	4.52×10^{-2}	4.73×10^{-2}	-5×10^{-4}
C	1.43×10^{-3}	"	4.28×10^{-2}	"	0.88×10^{-2}	4.31×10^{-2}	4.74×10^{-2}	2×10^{-4}
D	3.56×10^{-3}	"	3.64×10^{-2}	"	2.17×10^{-2}	3.69×10^{-2}	4.75×10^{-2}	2×10^{-4}
E	7.13×10^{-3}	"	2.57×10^{-2}	"	4.37×10^{-2}	2.58×10^{-2}	4.71×10^{-2}	5×10^{-4}

* *Calculated from the equation* $[CO_3^{--}]_F = [CO_3^{--}]_T - 3\,[UO_2^{++}]$.

If the corrections for the formation of the $UO_2(CO_3)_3^{\equiv}$ complex are not applied, and the titration results are interpreted in the usual way, each complexed CO_3^{--} ion is counted as two HCO_3^- ions since it is titrated only at the second end point. As shown in Table II, this leads to an erroneously high apparent concentration of HCO_3^-.

Effect of UO_2^{++} on the pH of the End Points

The progressive lowering of the pH of the second end point from 4.0 to 3.2 in the presence of uranium can be attributed to the hydrolysis of the uranyl ion (1), exemplified in the following equations:

$$UO_2^{++} + HOH \rightarrow UO_2(OH)^+ + H^+, \quad [9]$$

$$2UO_2^{++} + HOH \rightarrow (UO_2)_2OH^{+++} + H^+. \quad [10]$$

At the first end point, the uranium is still largely tied up in the form of carbonate complex ions, as shown earlier, and there is an insufficient concentration of free UO_2^{++} to reduce the pH appreciably from the normal value of 8.2 through similar hydrolysis.

The lowering of the pH of the second end point constitutes a further possible source of error in the determination of CO_3^{--} in the procedure commonly followed in routine determinations, i.e., that of titrating to predetermined constant end points (i.e. at pH of 8.2 and 4.0). The titration curves in Fig. 1 show that the second end point, estimated by this procedure, may be subject to considerable error when uranium is present.

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AN EFFECT OF POLYMERIZATION TEMPERATURE ON THE HUGGINS CONSTANT k' FOR A DIENE POLYMER[1]

By L. H. Cragg and G. R. H. Fern[2]

ABSTRACT

Samples of poly(butadiene-*co*-styrene) were polymerized in redox systems at 5° and −18° C., the recipes being such as to yield samples similar in composition, in percentage conversion, and in intrinsic vistex to those prepared at 50°, 40°, 30°, and 15° C. for an earlier study. The two samples were carefully fractionated and the viscosity functions $[\eta]$, β, and k' were determined for each of the fractions. k' was shown to be the same for all the fractions of a given sample—an indication that there is little or no branching at these low temperatures. Comparison with the earlier results reveals, however, that for unbranched species k' increases as the temperature of polymerization decreases, from $0.32_7 \pm 0.008$ at 50° to $0.37_0 \pm 0.005$ at −18° C. This increase in k' is attributed to an increase in the *trans*-1,4-content of the polymer.

INTRODUCTION

In earlier papers, evidence was presented that the Huggins slope constant k' (8) can be a useful measure of branching in high polymers (10, 5, 3, 4). When polymers containing branched species were carefully fractionated, the values of k' obtained with the fractions decreased regularly with the intrinsic viscosity of the fractions, finally reaching, with the lower fractions, a constant value $\underline{k}'$ characteristic of linear species of the same polymer (5, 3, 4). When samples of poly(butadiene-*co*-styrene), prepared at several different temperatures but alike in other respects, were studied in this way, the extent of branching was found to decrease with decreasing polymerization temperature. In other words, as the temperature of polymerization was lowered, more of the fractions were found to have a k' value equal to the base value $\underline{k}'$ common to unbranched species. In fact, when the polymerization temperature was 15°C., all of the fractions had the same k'. It was noted, however, that this constant value of k' at 15°C. was a little higher than the base value at 30°C. The increase was slight but too definite to be ignored. Accordingly, the investigation has been extended to still lower temperatures. This paper reports evidence, so obtained, that the effect is real and significant.

EXPERIMENTAL

The apparatus and procedure have already been described in detail (4). With the exception of the polymers, the materials (solvents, etc.) for this investigation were the same as those used earlier (4). The two *polymer* samples were prepared in much the same way as before, the only difference being the temperature of polymerization—5°C. and −18°C.—and the slight modifications (6) in the recipe that were required to make the intrinsic vistex, the percentage

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Contribution from the Department of Chemistry, Hamilton College, McMaster University, Hamilton, Ont. Financial assistance was received, in the form of a research grant, from the National Research Council of Canada, and, in the form of a scholarship, from the Research Council of Ontario.

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conversion, and the time of reaction nearly the same in these as in the earlier samples, namely, 1.9–2.0 dl./gm., 60–62%, and 17–18 hr., respectively.

RESULTS AND DISCUSSION

From each of the samples eight fractions of about equal size—each approximately 1/15th of the whole sample—were obtained by careful fractionation. For each of these fractions, values of $[\eta]$, β (the slope constant in the Fuoss equation $(\ln \eta_r)/c = [\eta] - \beta[\eta]^2 c$ (12)), and k' were obtained as before.

TABLE I

DATA FOR FRACTIONS OF THE RUBBER POLYMERIZED AT 5° C. (RF 17)
W = 5.048 gm., C = 1.009 gm./100 ml., W' = 4.64 gm.

Fraction	w (gm.)	w_x ($\times 10^2$)	$[\eta]$	β	k'
1	0.270_4	5.82	3.25_1	0.13_3	0.36_7
2	0.316_4	6.81	2.81_0	0.12_7	0.37_3
3	0.357_7	7.70	2.41_3	0.13_2	0.36_8
4	0.301_9	6.50	2.27_7	0.13_4	0.36_6
5	0.411_5	8.86	2.03_7	0.13_5	0.36_5
6	0.366_3	7.89	1.78_8	0.12_8	0.37_2
7	0.265_2	5.71	1.61_7	0.13_7	0.36_3
8	0.361_2	7.78	1.49_2	0.13_4	0.36_8
				Average	0.36_8

TABLE II

DATA FOR FRACTIONS OF THE RUBBER POLYMERIZED AT −18° C. (RF 18)
W = 4.949 gm., C = 0.990 gm./100 ml., W' = 4.55 gm.

Fraction	w (gm.)	w_x ($\times 10^2$)	$[\eta]$	β	k'
1	0.256_5	5.63	3.85_0	0.12_9	0.37_1
2	0.283_1	6.22	2.91_1	0.12_8	0.37_2
3	0.324_3	7.12	2.61_0	0.13_2	0.36_8
4	0.334_4	7.34	2.36_4	0.13_0	0.37_0
5	0.283_3	6.22	2.17_7	0.12_7	0.37_3
6	0.247_7	5.44	1.98_2	0.12_9	0.37_1
7	0.257_1	5.65	1.83_0	0.13_4	0.36_6
8	0.317_2	6.97	1.66_0	0.13_2	0.36_8
				Average	0.37_0

The results are given in Tables I and II. In these tables W is the total weight of the original rubber sample, W' is the weight of rubber in W (assuming 8% of the original sample to be materials extractable by the ethanol–toluene azeotrope), C is the original concentration of the solution from which the fractions were separated, w is the weight of a fraction, and w_x is its weight fraction.

All the fractions of the polymer prepared at 5°C. have the same value of k' within the limits of precision, namely, $0.36_8 \pm 0.005$. Similarly, for the fractions of the polymer prepared at −18°C. there is a characteristic value of k', $0.37_0 \pm 0.005$. There is, therefore, no evidence of branching in these samples and hence

the conclusion that there is little or no branching in cold rubber prepared at or below 15°C. receives additional support.

These two values of k', 0.36_8 and 0.37_0, do not differ significantly from each other, but they do differ significantly from the corresponding values for the polymers prepared at the higher temperatures. For example, k' for the 15°C. polymer is 0.34_3 (the values obtained for the individual fractions being 0.34_2, 0.35_2, 0.34_4, 0.34_3, 0.33_9, 0.34_0). The difference is, therefore, much too great to attribute to experimental error. That this increase in k' with decreasing temperature of polymerization is a real effect is more evident still when all the data available are considered, as in Fig. 1, where k' is plotted against the polymeriza-

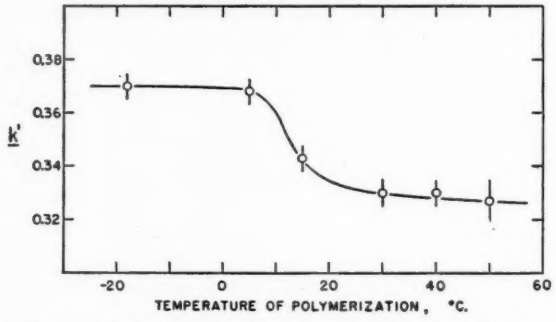

FIG. 1. Variation of $\underline{k}'$ with temperature of polymerization.

tion temperature. (For each of the higher temperatures at which branched species are formed, the value of k' plotted is $\underline{k}'$, the "base value" characteristic of the unbranched species (4). In the figure the length of a vertical line indicates roughly the precision of the $\underline{k}'$ value.)

Since all viscosity measurements were made in the same solvent, benzene, and at the same temperature, 25.0°C., the observed differences in k' must be due to differences in the polymer molecules themselves. They cannot be due to changes either in molecular weight or in molecular weight distribution, because the measurements of k' were made on fractions, and in a given sample k' was independent of molecular weight. They cannot be due to branching, for it has been shown that branching decreases with decreasing polymerization temperature, and an increase in k' would indicate an increase in branching. Moreover, all the evidence shows that branching does not affect all fractions to the same degree (3, 4, 5).

They must, therefore, be due to changes in the structure of the molecular chain. But again we can eliminate most of the possibilities. Differences in composition of the molecules—i.e., in the ratio of butadiene to styrene units—cannot be responsible. Beatty and Zwicker (2) have shown that for such polymers as these the proportion of styrene is the same, within experimental error, for all the fractions of a polymer sample and differs only slightly for polymers prepared at different temperatures, averaging 21.7 at 50°C. and 22.8 at −18°C. Yet, the fractions of a sample prepared at 0°C. from a recipe in

which the monomer ratio was 85:15 gave k' values of 0.037_0,* which is just what would be expected of a sample prepared at that temperature from a 72 : 28 recipe, and certainly this change in monomer ratio would cause a greater difference in the proportion of styrene in the molecules than would the change in polymerization temperature.

There remain three other possibilities, each related to a form of chain isomerism. In any vinyl polymer a type of isomerism analogous to rotational isomerism can occur (11). Species with different spatial arrangements of successive substituent groups on the polymethylene chain may have widely differing properties (13). Indeed, the decrease in k' with increasing polymerization temperature that was observed in polystyrene by Alfrey, Bartovics, and Mark (1) was attributed by Huggins (9) to such differences in chain configuration. This cannot, however, be the cause of the changes in k' that we have observed. In the polymer samples which we studied the proportion of bound styrene would be about 22% by weight (2, 7) so that successive styrene residues would, on the average, be separated from each other by six or seven butadiene residues. Hence, the arrangement of substituents in the styrene portions of the chain would have little or no effect on the properties of the polymer. In such polymers the types of isomerism found in diene polymers (like polybutadiene, for example) would, on the other hand, be quite significant. These are the structural isomerism resulting from the possibility of either 1,4 or 1,2 additions of the butadiene, and the geometrical (*cis–trans*) isomerism made possible by the double bond left in each 1,4 diene unit in the chain. Infrared analysis has shown that in polymers of the type we have studied the ratio of 1,4 to 1,2 units changes very slightly with the temperature of polymerization; according to Hart and Meyer the 1,2-content is 22% at 50°C. and 20% at −18°C. (7). By contrast, the ratio of *trans*-1,4 to *cis*-1,4 units increases significantly with decreasing polymerization temperature; Hart and Meyer found the *trans*-1,4-content to be 65% at 50°C. and 84% at −18°C. (7).

Of the variables known to be affected by a change in polymerization temperature, *cis–trans* isomerism, then, would seem to be the only possible source of the differences in k' that we have observed. This, of course, does not prove that it is the source. But at least one would expect that any effect on k' of an increase in the *trans*-1,4-content would be an increase in k'. More of the *trans* configuration would mean a stiffer and hence less compressible molecule. (For example, gutta percha, which is largely the *trans* isomer of polyisoprene, is much harder and less elastic than natural rubber, the *cis* form.) And of two polymer species in solution, the one whose molecules are less compressible should give the higher value of k'.

Summing up, then, we can say that a change in the temperature of polymerization of poly(butadiene-*co*-styrene) causes a parallel change in k' and in *trans*-1,4-content of the polymer chains and, further, that it is reasonable to attribute the change in k' to the isomeric change.

ACKNOWLEDGMENTS

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* *Unpublished data, from this laboratory.*

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ON KHELLACTONE[1]

By E. Späth,[2] W. Gruber,[3] and O. Matzke[4]

ABSTRACT

A hitherto unknown minor constituent of the seeds of *Ammi visnaga* Lam. is converted under the influence of 5% alcoholic potassium hydroxide into a compound which seems to be a coumarin. Treatment with methyl-alcoholic alkali yields a substance $C_{15}H_{16}O_5$, whereas treatment with ethyl-alcoholic alkali gives a substance $C_{16}H_{18}O_5$. Dehydrating agents convert both optically active compounds into the same optically inactive dehydration product $C_{14}H_{12}O_4$ (Substance A). The further investigations carried out with Substance A were: oxidation, after methylation, which yielded α-hydroxyisobutyric acid; fusion with potassium hydroxide, which gave phloroglucinol. On treatment with diluted caustic alkalies two definite products, $C_{10}H_8O_5$, probably an acid, and $C_{11}H_8O_4$, a ketone, were obtained.

The main constituents of the umbelliferous plant *Ammi visnaga* Lam., khellin, visnagin, and chellol-glucoside, have been thoroughly investigated chemically as well as pharmacologically (3, 4, 5, 6) but only a few investigations of minor constituents have been published (3).

Recently Steinegger (7) extracted the seeds of *Ammi visnaga* Lam. cultivated in Switzerland. He separated 1,2- and 1,4-pyrones carefully and found a previously unknown coumarin $C_{17}H_{18}O_6$, m.p. 187–188°. Some time ago we worked up the ether extract of Egyptian Chellah seeds and isolated a new substance, which also seemed to be a coumarin. Owing to difficulties in getting the necessary raw material we could not elucidate the structure of this coumarin, khellactone, completely. However, since khellactone has a vasodilator action similar to that of khellin, the results are published in order to encourage further investigations.

The crude ether extract was washed with 0.5% aqueous potassium hydroxide to remove free acids and phenols and then evaporated. The residue underwent the so-called "lactone separation", in which lactones, esters, etc. are converted into salts of the corresponding acids by treatment with alcoholic potassium hydroxide after which the acids are again set free with hydrochloric acid. Hydroxy acids, originating from lactones, give ring closure when treated with hydrochloric acid and thus form neutral lactones which are easily separated from acids derived from esters, e.g. glycerides. The diluted and acidified saponification mixture was allowed to stand for several hours and then extracted with a large amount of ether. To avoid emulsification, the ethereal solution was percolated with 0.5% aqueous potassium hydroxide solution until an acidified sample of the percolate showed no more precipitation. The residue on evaporation of the ether was redissolved in ether, percolated a second time in the same way, and finally purified by vacuum distillation and recrystallization; yield 0.06%, white crystals, dimorphous, lower m.p. 137–138°C., higher m.p. 167–168°C.

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Contribution from the Second Chemical Institute, University of Vienna, Austria; taken from the thesis presented by O. Matzke in partial fulfillment of the requirements for the Ph.D. degree.
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Either form can be obtained from the melt by using the appropriate seed crystal. The analysis suggested a formula $C_{16}H_{18}O_5$; methoxyl determination (Zeisel's method) indicated one OCH_3-group. Determination of active hydrogen (Zerewitinoff's method) at room temperature showed about one-half hydrogen atom; but at 100°C. the result indicated one active hydrogen atom. Micro-hydrogenation (1) (in glacial acetic acid with palladium black) indicated one double bond; optical activity: $[\alpha]_D^{20°} = -37.1$.

Since it was probable that the active hydrogen belonged to an alcoholic hydroxy-group we tried to introduce a second double bond into the molecule by dehydration. Dehydration could be effected with phosphorus pentoxide, and with still better yields using hydrobromic acid in glacial acetic acid. The resulting derivative (Substance A) was also dimorphous: lower m.p. 147°C., higher m.p. 157–158°C. It was not optically active. The presence of a second double bond in the molecule was proved by microhydrogenation (1). With *p*-nitro-phenylhydrazine, A gave a derivative, m.p. 223–224°C., which we consider to be a *p*-nitrophenylhydrazone. Finally the results of the combustion suggested a gross formula $C_{14}H_{12}O_4$, obviously originating from the original compound $C_{16}H_{18}O_5$ by loss of C_2H_6O.

This ethyl group was probably introduced during the lactone separation, owing to the treatment with ethyl-alcoholic potassium hydroxide, and the effect of the dehydrating agent was to split it off again. In this case the alkoxyl group, found by Zeisel analysis, should be an ethoxyl instead of a methoxyl group. In order to identify it, the ester of 3,5-dinitrobenzoic acid was synthesized from the silver salt of the acid and the alkyl iodide formed in the Zeisel apparatus through cleavage of the khellactone, following the method of Furter (2). The resulting product was the ethyl ester of the 3,5-dinitrobenzoic acid.

A fresh batch was then worked up using methyl-alcoholic potassium hydroxide and gave another coumarin, m.p. 156–158°C., in 0.18% yield (based on the dry weight of seeds). The Furter method for identifying the alkoxyl group gave the methyl ester of 3,5-dinitrobenzoic acid. Methoxyl analysis (Zeisel-Vieböck method) and combustion gave values which agreed unequivocally with the gross formula $C_{15}H_{16}O_5$.

We propose for this compound the name of methyl khellactone and for the product of the first extraction the name of ethyl khellactone. These names for the two derivatives of the genuine substance are justified by the fact that both yielded Substance A when boiled with hydrobromic acid in glacial acetic acid. It is therefore clear that the alkoxyl groups were introduced during the isolation. We did not have enough "Chellah" seeds to carry out a new extraction so as to isolate the unaltered khellactone.

While the treatment with the alcoholic alkali and the dehydration with strong acids may have caused drastic changes in the khellactone molecule, nevertheless we have carried out a few experiments in the hope of getting some indications of the basic structure of the molecule.

After fusing ethyl khellactone with a mixture of sodium and potassium hydroxide, phloroglucinol was isolated, characterized as the triacetate and compared with an authentic sample.

Again Substance A was methylated with dimethyl sulphate and potassium hydroxide and to the resulting oil in alkaline solution was added 1% potassium permanganate solution in small portions at a temperature of 30°C. until the red color was stable for some time. About 5.5 moles of oxygen was consumed. α-Hydroxyisobutyric acid was isolated and characterized as the phenylhydrazide.

Treatment of Substance A with alkali gave very interesting results. With 10% aqueous or alcoholic alkaline solutions the reaction was complex, but with 0.5–5.0% alkaline solutions in the cold two substances were formed (B and C).

Substance B, obtained by high vacuum distillation (0.005 mm. Hg, 170°C. air bath), consisted of compact prisms, m.p. 257–258°C.; easily soluble in dilute alkali bicarbonate solutions. These solutions showed strong blue fluorescence whereas the solutions in concentrated sulphuric acid showed a violet fluorescence. With ferric chloride, Substance B showed a color reaction similar to that of salicyclic acid. The combustion values agreed best with a formula $C_{10}H_6O_5$; no alkoxyl group. However, Substance B is not identical with umbelliferone-6-carboxylic acid or with umbelliferone-8-carboxylic acid, as shown by depression of the mixed melting points. It could not be decarboxylated with quinoline and copper powder (Naturkupfer "C", Kahlbaum).

Substance C was also isolated by high vacuum distillation (0.005 mm. Hg, 200° air bath), intense yellow prisms, m.p. 264°C. (dec.). It was soluble only in caustic alkali and the solutions did not show any fluorescence. With *p*-nitrophenylhydrazine in dilute acetic acid it yielded immediately a *p*-nitrophenylhydrazone. The combustion values were compatible with a gross formula $C_{11}H_8O_4$; no alkoxyl group present.

Compounds B and C are probably related in the same way as an *o*-hydroxyketone and the corresponding acid, formed in the alkaline cleavage of a γ-pyrone. Further investigations with these two cleavage products could not be carried out however, since the amounts on hand were too small.

EXPERIMENTAL

Melting points were made on the Kofler block and are corrected.

Extraction and Isolation of Ethyl Khellactone

Two thousand grams of finely ground seeds of *Ammi visnaga* Lam. were extracted with ether for 100 hr.; the ether was then decanted and made up to 2000 ml. with ether and percolated with 0.5% aqueous potassium hydroxide. The ethereal solution, now free from substances soluble in alkali, was evaporated to dryness and the lactone separation applied on the residue which was then treated with 450 ml. of 5.0% ethyl-alcoholic potassium hydroxide. The mixture was allowed to stand for two hours with occasional shaking, then diluted with 2.5 liters of water and extracted with ether. The aqueous layer was acidified to Congo red with hydrochloric acid and kept for one day after which it was exhaustively extracted with ether and again percolated with 0.5% aqueous potassium hydroxide. Evaporation of the ether left a yellowish oil with a sweet smell; the lactone separation was repeated on this oil, using only 150 ml. of 5.0% ethyl-alcoholic potassium hydroxide. The product was distilled *in vacuo* (0.03

mm. Hg, 160–180°C. air bath) giving a crude yield of 1.268 gm. Purification by recrystallization from ether–petroleum ether under pressure gave long needles, m.p. 166–167°C. On cooling, the substance solidifies at 165°C., softens again at 150°C., and resolidifies at 138–137°C. On careful heating of the melting point apparatus both melting points, 137–138°C. and 166–167°C., can be observed. For $C_{16}H_{18}O_5$ calc.: C, 66.20, H, 6.23, OC_2H_5, 15.51%; found: C, 66.47, H, 6.28, OC_2H_5, 15.68%.

Dehydration to Substance A

(*a*) *With Phosphorus Pentoxide*

Ethyl khellactone (38.4 mgm.) was mixed with phosphorus pentoxide (40 mgm.) and distilled *in vacuo* (0.001 mm. Hg, 170°C. air bath). Yield: 8.4 mgm. of an oil which solidified immediately. Purification was by recrystallization from ether until a constant melting point of 157–158° was reached. This substance was dimorphous: lower m.p. 147°.

(*b*) *With Hydrobromic Acid and Glacial Acetic Acid*

Ethyl khellactone (30.0 mgm.) was refluxed for 35 min. with 0.06 ml. hydrobromic acid and 0.04 ml. glacial acetic acid at 130°, the mixture then diluted with 2 ml. water and extracted with ether. The ethereal layer was shaken with sodium bicarbonate solution and finally the ether was evaporated. The residue was sublimed *in vacuo*, 0.002 mm. Hg, 145–150°C. (air bath); yield: 7 mgm. After recrystallization from ether under pressure the melting point was 156–158°C.; the mixed melting point of this substance and the one prepared after the phosphorus pentoxide method showed no depression. For $C_{14}H_{12}O_4$ calc.: C, 68.84, H, 4.95%; found: C, 68.85, H, 5.08%.

Identification of the Alkoxyl Group

Ethyl khellactone (7.8 mgm.) was boiled with hydriodic acid ($d = 1.7$) in a Zeisel apparatus, following the method of Furter. The alkyl iodide was carried over into a cooled ethereal suspension of the silver salt of 3,5-dinitrobenzoic acid. After sealing the receiver and heating for two hours in the water bath the ethereal solution was decanted from the silver halide and the solution washed with dilute aqueous potassium hydroxide, hydrochloric acid, and finally with water. After evaporation of the ether the residue was distilled in a high vacuum and had a melting point 91–92°C., not depressed by admixture with synthetic ethyl 3,5-dinitrobenzoate.

Extraction and Isolation of Methyl Khellactone

This was carried out as described above for the preparation of ethyl khellactone, except that for the lactone separation 5% methyl-alcoholic potassium hydroxide solution was used instead of 5% ethyl-alcoholic solution. After purification by recrystallization and sublimation *in vacuo* (0.005 mm. Hg) the melting point was 156–158°C. and the mixed melting point with ethyl khellactone showed depression. This method of isolation, using methanol instead of ethanol, gave much better yields: 200 gm. of the dry seeds gave 0.4 gm. methyl khellactone while 2000 gm. of the dry seeds gave only 1.268 gm. of ethyl khellactone.

For $C_{15}H_{16}O_5$ calc.: C, 65.21, H, 5.84, OCH_3, 11.22%; found: C, 65.48, H, 5.93, OCH_3, 11.05, 11.33%.

Identification of the Methoxyl Group

This analysis was carried out according to Furter's method, as described above. The ester obtained had a melting point of 103–104°C., which was not depressed by admixture with an authentic sample of methyl 3,5-dinitrobenzoate. (The mixed melting point with the ethyl 3,5-dinitrobenzoate showed a heavy depression.)

Cleavage of the Methyl Khellactone with Hydrobromic Acid and Glacial Acetic Acid

Methyl khellactone (290 mgm.) was refluxed for 30 min. with 0.6 ml. of 48% hydrobromic acid and 0.6 ml. of glacial acetic acid. The reaction mixture was worked up as described for ethyl khellactone. The reaction product melted at 157–158°C. and showed no depression in a mixed melting point with Substance A, derived from ethyl khellactone; yield 188 mgm., i.e. 67%. For $C_{14}H_{12}O_4$ calc.: C, 68.84, H, 4.95%; found: C, 68.80, H, 4.71%.

p-Nitrophenylhydrazone of Substance A

Substance A (10 mgm.) was dissolved in 1.5 ml. of 80% acetic acid and a solution of 9 mgm. of *p*-nitrophenylhydrazine in 1.5 ml. of dilute acetic acid added. The *p*-nitrophenylhydrazone separated immediately and was collected after two hours and purified by recrystallization from 5% acetic acid, m.p. 233–235°C. (decomp.).

Microhydrogenation of Substance A

Substance A (5.716 mgm.) was hydrogenated in 5 ml. of glacial acetic acid with 12.32 mgm. palladium black (735 mm., 19.7°C.). After four and one-half hours two molecules of hydrogen were absorbed (= 1160 $mm.^3$). The resulting tetrahydro-product was a colorless oil, which did not crystallize. The amount was too small for further investigations.

Fusion of Ethyl Khellactone with Potassium Hydroxide

To a homogeneous melt of 10 gm. potassium hydroxide and 10 gm. sodium hydroxide, 64 mgm. of khellactone was added and the mixture heated for 10 min. with stirring. After cooling, the reaction cake was dissolved in water, acidified with hydrochloric acid, and extracted with ether for 18 hr. After evaporation of the ether and distillation *in vacuo* (0.05 mm. Hg) the main product (130–170°C. air bath) was directly converted into the acetyl product by boiling with acetyl chloride. Purification of this product by distillation *in vacuo* (0.05 mm. Hg) gave the main product at 120–130° (air bath) consisting of white crystals, m.p. 96–98°, undepressed when mixed with a synthetic specimen of "phloroglucinol-triacetate".

Oxidation of Substance A with Potassium Permanganate

Substance A (97 mgm.) was dissolved in 4 ml. 5% aqueous potassium hydroxide and methylated with 1.0 ml. dimethyl sulphate and 5 ml. 10% aqueous potassium hydroxide in the usual way. Finally the reaction mixture was slightly acidified, extracted with ether, the ethereal solution exhaustively

washed with 0.5% potassium hydroxide, the yellow alkaline solution acidified and extracted with ether. Evaporation of the ether and distillation *in vacuo* (0.05 mm. Hg) yielded 73 mgm. of a yellowish oil. This oil was dissolved in 15 ml. of 1% aqueous potassium hydroxide (the solution showed an intense yellow color), the volume brought to 50 ml., and at a temperature of 30°C. 1% aqueous potassium permanganate solution was added dropwise. After addition of 15.5 ml. of the permanganate solution (5.5 moles of oxygen) the violet color was stable for about 30 min. The oxidation mixture was worked up as usual and the resulting product finally fractionated *in vacuo* (0.05 mm. Hg): main product (4 mgm.) at a temperature of 70–85°, white, hygroscopic crystals, m.p. 73–75°C.; mixed m.p. with synthetic α-hydroxyisobutyric acid 73–75°C.

Since the melting point as well as the mixed melting point of the phenylhydrazides from the synthetic acid and our degradation product were identical, the degradation product was proved to be α-hydroxyisobutyric acid.

Alkaline Cleavage of Substance A

(*a*) *With 10% Alcoholic Potassium Hydroxide*

Substance A was completely decomposed, so that on acidification only resinous products were obtained.

(*b*) *With 5% Alcoholic Potassium Hydroxide*

Substance A (95 mgm.) was kept for two hours with 5% methyl-alcoholic potassium hydroxide (orange-yellow solution). The reaction mixture was then diluted with 250 ml. of water, acidified with hydrochloric acid (the color of the solution changed to yellow), kept overnight, and exhaustively extracted with ether. The ethereal solution was treated with 500 ml. of 0.5% potassium hydroxide. There was no residue on evaporation of the ether; therefore the yellow, alkaline solution was acidified to Congo red and extracted with ether. The residue was distilled *in vacuo* (0.005 mm. Hg). There were two distinct fractions; after a forerun (to 150°C.):

(1) Substance B: 160–180° (air bath), white crystals (30 mgm.); after recrystallization from ether under pressure, m.p. 256–258°. For $C_{10}H_6O_5$ calc.: C, 58.26, H, 2.93%; found: C, 58.56, H, 2.93%.

M.p. of umbelliferone-6-carboxylic acid 265–268°C.; mixed m.p. with the above substance 205–215°C.

M.p. of umbelliferone-8-carboxylic acid (dimorphous): lower m.p. 235–237°C., higher m.p. 258–260°C.; m.p. of the mixture of our degradation product with the higher melting form 220–225°C.

(2) Substance C: 190–210°C. (air bath), yellow crystals (45 mgm.); purification by recrystallization from ether under pressure, yellow plates, m.p. 263–265°C. (decomp.). For $C_{11}H_8O_4$ calc.: C, 64.72, H, 3.95%; found: C, 64.83, H, 4.07%.

(*c*) *With 0.5% Alcoholic Potassium Hydroxide*

Substance A yielded the same two products as described under (*b*); their identity was established by melting points and mixed melting points.

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THE COMPRESSIBILITY OF GASES

VII. ARGON IN THE TEMPERATURE RANGE 0–600° C. AND THE PRESSURE RANGE 10–80 ATMOSPHERES[1]

By E. Whalley,[2] Y. Lupien, and W. G. Schneider

ABSTRACT

The virial coefficients of argon have been measured in the temperature and pressure range described. "Best" values of the virial coefficients have been computed from all measurements reported in the literature.

The method of measurement has been adequately described previously (6, 11, 12, 15). The two pipettes had volumes of about 220 and 56 ml. The temperature was controlled to ± 0.005° and before each measurement of the pressure was maintained to ± 0.002° for 15 min. by manual adjustment of the control circuit. To maintain 0°C., it was found easier to use a cooling coil in the bath and an intermittent heater than to use ice.

The argon used in the first runs was "commercial argon" supplied by Linde Company and was stated to contain a minimum of 99.8% of argon and a maximum of 0.2% of nitrogen having a purity of 99.99%. The nitrogen was removed by passing the argon slowly several times over heated magnesium until mass spectrometer analysis showed that no nitrogen (< 0.02%) was present. Subsequently, we used "refined argon" stated to contain less than 5 p.p.m. each of hydrogen, oxygen, nitrogen, and carbon dioxide, and less than 3 p.p.m. of water. Runs performed with the two grades at the same temperature showed no difference in compressibility. Frequent checks on the purity of the gas were made during the runs by mass spectrometer analyses. These were kindly performed by Dr. F. P. Lossing and co-workers.

ANALYSIS OF RESULTS

The pressure of the gas in the large pipette was measured; then the gas was expanded into the small pipette and the pressure measured again. The pressures P_1 and P_2 before and after expansion are related by the equation

$$[1] \qquad \frac{P_1}{P_2} = N + (N-1)\frac{B}{A}P_1 + \left(N - \frac{P_2}{P_1}\right)\frac{C}{A}P_1^2 + \ldots$$

if the equation of state of the gas can be written

$$[2] \qquad PV = A + BP + CP^2 + \ldots$$

and N is the ratio of the total volume of both pipettes to the volume of the large pipette. The measured values of P_1/P_2 and P_1 were fitted to Equation [1] by the method of least mean squares described below, and the values of the parameters N, B/A, and C/A determined. Sufficient terms were used that the minimized sum of the squares of the deviations was not appreciably reduced

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by the addition of another term in the next higher power of P_1 and the deviations were randomly distributed. None of the measurements warranted a higher term than P_1^2, and from 400° upwards the term in P_1^2 was insignificant.

If the isotherms were not linear the N value was determined by a series of expansions with helium which gives a linear isotherm. Equation [1] could then be transformed to

$$[3] \qquad \frac{P_1/P_2 - N}{P_1} = (N-1)\frac{B}{A} + \left(N - \frac{P_2}{P_1}\right)\frac{C}{A}P_1 + \ldots$$

which is linear if Equation [2] is not of higher order than quadratic. The results were fitted to this equation by a method similar to that described below. The mean of the B/A and C/A values obtained from Equations [1] and [3] was used. In the final runs the B/A and C/A values determined by the two methods agreed within experimental error.

The method used in fitting the measured values of P_1/P_2 and P_1 to Equation [1] is different from that used in previous papers and has the advantages that the amount of work is considerably lessened, the standard errors of B and C are readily calculated, and the approximation that $(N - P_2/P_1)$ is constant is not made. We therefore include a brief description of the method used. Equation [1] was written in the form

$$[4] \qquad y = a + (a-1)bx + (a - 1/y)cx^2,$$

where

$$[5] \qquad \begin{aligned} y &= P_1/P_2,\ x = P_1, \\ a &= N,\ b = B/A,\ c = C/A. \end{aligned}$$

It was assumed that x and y are independent measurements. This is not true as is evident from their definitions, but is sufficiently accurate for our purposes. To check this, the 600°C. runs were fitted to the equation

$$[6] \qquad \frac{1}{P_2} = \frac{N}{P_1} + (N-1)\frac{B}{A}$$

by the method of least mean squares. There was no significant difference in the values of either N or B/A obtained by using Equations [4] and [6].

The procedure for least mean squares fitting is that described by Deming (3). Approximate values of a, b, and c, say a_0, b_0, and c_0, were obtained from previous measurements of the compressibility or from the graph of x vs. y. If

$$[7] \qquad \begin{aligned} a &= a_0 - \alpha, \\ b &= b_0 - \beta, \\ c &= c_0 - \gamma, \end{aligned}$$

the normal equations for computing α, β, and γ are written with detached coefficients as follows:

$$[8] \qquad \begin{array}{cccc} \alpha & \beta & \gamma & = \quad 1 \\ \sum \frac{F_a^2}{L} & \sum \frac{F_aF_b}{L} & \sum \frac{F_aF_c}{L} & \sum \frac{F_aF_0}{L} \\ \sum \frac{F_aF_b}{L} & \sum \frac{F_b^2}{L} & \sum \frac{F_bF_c}{L} & \sum \frac{F_bF_0}{L} \\ \sum \frac{F_aF_c}{L} & \sum \frac{F_bF_c}{L} & \sum \frac{F_c^2}{L} & \sum \frac{F_cF_0}{L} \end{array}$$

Each row but the first is a normal equation and each term is to be multiplied by the parameter in the first row.

The various quantities used in Equation [8] are:

$$[9] \qquad \begin{aligned} F &= y - a - (a-1)bx - (a - 1/y)cx^2, \\ F_0 &= y - a_0 - (a_0 - 1)b_0x - (a_0 - 1/y)c_0x^2, \\ F_a &= \partial F/\partial a, \text{ etc.}, \\ L &= (F_x^2/w_x) + (F_y^2/w_y), \end{aligned}$$

and w_x and w_y are the weights of x and y. Since F_x is of the order $10^{-3} - 10^{-4}$ and F_y is of the order unity and w_x and w_y are of the same order, F_x^2/w_x is negligible in comparison with F_y^2/w_y. F_y differs from unity by a maximum of about 1%. Since we are calculating the small quantities α, β, and γ four or five figures are sufficient in the normal equations, and since the factor F_y^2 appears as a divisor in all the square and cross products of F_a, etc., it is quite safe to neglect it. The sensitivity of the piston gauge does not vary much with pressure and we gave each value the same weight. Consequently the L terms are constant and cancel from the normal equations. This considerably reduces the computation required. The minimized sums of squares and the standard error of the parameters are readily calculated by the method described by Deming (3).

The value of A at temperature T is defined by

$$[10] \qquad A = A_0T/273.16$$

where A_0 is the value of A at 0°C. A_0 is obtained from the identity that if the unit of volume is the normal volume or amagat, then at one atmosphere pressure and 0°C.

$$[11] \qquad PV = 1 = A_0 + B_0 + C_0.$$

B_0/A_0 and C_0/A_0 are known from the 0°C. isotherm so A_0 is readily obtained. The standard error of A_0 was obtained from the relation

$$[12] \qquad \sigma(A_0) = \{[\sigma(B_0/A_0)]^2 + [\sigma(C_0/A_0)]^2\}^{\frac{1}{2}},$$

where σ denotes the standard errors of the quantities within the round brackets.

RESULTS

The results are given in Table I. The unit of pressure is the standard atmos-

TABLE I

VIRIAL COEFFICIENTS OF ARGON, N.R.C. VALUES

T, °C.	A	S.E. of 10^6A	10^3B, amagat/mole	10^6C, amagat/mole/atm.	No. of expansions	S.E. of $10^5 \frac{P_1}{P_2}$
0	1.000999	10	−1.001 ± .010	2.37 ± .07	33	7.1
50	1.184225	12	−0.500 ± .011	1.86 ± .11	41	9.5
100	1.367450	14	−0.194 ± .004	1.59 ± .04	37	2.6
150	1.550676	16	+0.045 ± .006	1.27 ± .06	41	3.9
200	1.733902	17	0.236 ± .007	0.96 ± .05	38	3.1
300	2.100353	21	0.481 ± .011	0.61 ± .08	44	3.3
400	2.466805	25	0.703 ± .005		27	6.2
500	2.833258	28	0.793 ± .006		35	5.6
600	3.199708	32	0.870 ± .006		33	4.9

phere and the unit of volume the normal volume. The normal volume V_n is RT_0/A_0 and is, from our measurements, 22.3916 ± .0022 l./mole or 22,392.2 ± 2.2 cc./mole.

COMPARISON WITH PREVIOUS RESULTS

The P-V-T relations for argon have been measured by Onnes and Crommelin (9) at Leiden; Holborn, Schultze, and Otto (4, 5, 10) at Berlin; Masson, Dolley, and Tanner (7, 13) at Durham; and Michels, Wijker, and Wijker (8) at Amsterdam at high pressures; and by Baxter and Starkweather (1) at low pressures. In Tables III, IV, and V, we compare the various values of A_0, B, and C, respectively, obtained by various workers.

The Leiden data agree badly with the other measurements, apparently because of "side-trapping in one limb of the piezometer after measuring the volume at one atmosphere pressure" (2, 14). They are therefore omitted from this discussion.

The Berlin data cover the temperature range −100° to +400°C. and pressure range 0–100 atm., and measurements were made up to 200 atm. at 0° and 100°C. We have not used the latter results since our measurements are only to 80 atm. As pointed out by Cragoe (2) the results were not worked up correctly and we have considered it worth while to recalculate from the measurements. We have given a rather extended discussion of the Berlin data since the PVT properties of a number of gases below 0°C. and above 150°C. are known only from these measurements.

The experimental procedure was briefly as follows. An unknown mass of gas was confined in a known volume V at a known high pressure and at a known temperature near (± 0.5°) the temperature at which PV was required. The gas was then expanded into a known volume V_u at a pressure P_u of about 1 atm. The following corrections were applied.

1. To V− (i) Correct to round values of the temperature 0°, 50°, etc.
   (ii) Correct for dead space not at temperature of measurement.
   (iii) Correct for pressure variation of dead space volume since the pressures inside and outside the dead space were not equal.

The sum of these corrections is about 0.3–0.4% of the total volume and it is difficult to decide whether the corrections were made by assuming the gas to be ideal.

2. To V_u− (i) Dead space.
   (ii) P_u and V_u were measured at about 16°C. and V_u corrected to be the volume the gas would occupy at 0°C. and pressure P_u.

Again it is difficult to decide whether the corrections were made by assuming the gas to be ideal. For lack of knowledge we have assumed that these corrections were applied correctly, i.e. using real values for PV. If this were not done, the results would be appreciably affected.

Holborn and Otto have assumed that

$$P_u V_u = P_0 V_0 \tag{13}$$

where V_0 is the volume that the gas in the piezometer would occupy at the unit pressure P_0 which is 1 m. of mercury. In fact, P_u is usually in the range 65–85

cm. of mercury and because the gas is not ideal Equation [13] cannot be assumed correct. We have calculated P_0V_0 where possible on the basis $P_0 = 1$ atm. making allowance for the difference between P_u and 1 atm. For the 0°, 50°, 100°, 150°, and 200° isotherms this correction was not possible since the values of P_u and V_u were not quoted. We have therefore assumed that P_u was near 1 atm. and that P_uV_u is very close to P_0V_0 for $P_0 = 1$ atm. If the values of P_u are similar to those quoted for other runs, this will probably not be a bad approximation.

Then

$$PV/P_0V_0 = A + BP + CP^2 + \ldots . \qquad [14]$$

We analyzed the data according to this equation. At 0°C., by definition of the unit of volume, Equation [11] holds so that

$$\frac{PV/P_0V_0 - 1}{P - 1} = B_0 + C_0(P + 1). \qquad [15]$$

A graph of $\frac{PV/P_0V_0 - 1}{P - 1}$ vs. $P + 1$ gives B_0 and C_0. Least mean squares were not used since we could not correct P_uV_u. The A_0 obtained was used to calculate A at any other temperature. The use of the "best" value of A_0 (see below) would have made very little difference to the value of B and C. For temperatures other than 0°C. the data were evaluated from the equation

$$(PV - A)/P = B + CP. \qquad [16]$$

Least mean squares were used for −100°, −50°, 300°, and 400°C. since at these temperatures we could correct P_uV_u to P_0V_0. At other temperatures B and C were evaluated graphically. The 300°C. isotherm gave a very strongly curved line, indicating that the A used was not correct. Using Equation [14] to determine the best values for A, B, and C by least mean squares, we found $A = 2.10277$. The A_0 $(= AT_0/T)$ value derived from this is 1.00215 which is wildly wrong. If we assume that $A_0 = 1.00096$ then we find that the temperature of the runs must have been 300.7°C. This seems the only reasonable explanation of the 300°C. measurements.

TABLE II

VIRIAL COEFFICIENTS OF ARGON FROM THE BERLIN DATA

T, °C.	$10^3 B$		$10^3 C$	
	Berlin analysis	Our analysis	Berlin analysis	Our analysis
−100	−2.873	−2.850 ± .014	−10.3	−10.2 ± .6
− 50	−1.688	−1.643 ± .007	+0.79	−0.845 ± .26
0	−0.986	−0.96	2.37	+2.09
50	−0.492	−0.465	1.79	1.50
100	−0.191	−0.167	1.61	1.35
150	+0.052	+0.088	1.24	0.90
200	0.209	0.233	1.12	1.03
300.7	0.501	0.439 ± .003	0.46	0.90 ± .26
400	0.683	0.669 ± .007		0.19 ± .22

The original values of Holborn and Otto, converted directly to $P_0 = 1$ atm., are compared with the results of our analysis in Table II and the corrected values are included in Tables III, IV, and V.

The Durham data were obtained over the temperature range 25–174°C. and pressure range 5–125 atm. The value of $A_0 = 1.00098$ obtained by Holborn and Otto was used. The measured PV values are not quoted; the virial coefficients are given together with the mean deviation of PV.

The Amsterdam data were obtained over the temperature range 0–150°C. and pressure range 20–2900 atm. The data up to 80 atm. were fitted to the equation

$$PV = A_\rho + B_\rho\rho + C_\rho\rho^2, \tag{17}$$

where ρ is the density in amagat units. Exact comparison of this equation with Equation [2] is difficult as they are not exactly equivalent. If both series are carried to a large enough number of terms they become equivalent and the following relationships hold,

$$\begin{aligned} A &= A_\rho, \\ B &= B_\rho/A_\rho, \\ C &= (A_\rho C_\rho - B_\rho^2)/A_\rho^3, \text{ etc.} \end{aligned} \tag{18}$$

But if the series are carried only to the square term, these relationships cannot hold exactly. In a later paper concerned with the intermolecular potential of argon, we shall be interested in the relation between the constants of Equations [2] and [17] since the constants in Equation [17] are those usually used in discussing the intermolecular potential. It is therefore worth while examining in some detail the validity of Equations [18] when Equations [2] and [17] are restricted to quadratic.

Michels' values of A_ρ, B_ρ, and C_ρ for argon at 0°C. up to 80 atm. were converted to A, B, and C using Equations [18]. The virial coefficients do not, unfortunately, have standard errors assigned to them and are quoted to six figures; we have omitted the last three as these are not significant. The original data of Michels' up to 80 atm. which contain the measured values of PV and P were fitted to Equation [2] by the method of least mean squares and the following results were obtained.

	A	10^3B	10^6C
Equations [18]	1.00097	− 0.956	1.62
Equation [2]	1.000963	− 0.965 ± .007	2.02 ± .02

From the measurements at 150°C. the following results were obtained.

	A	10^3B	10^6C
Equations [18]	1.55065	0.062	1.13
Equation [2]	1.550620	0.0698 ± .0013	0.98 ± .03

The A values at 150°C. were obtained from the respective A_0's by using Equation [10]. It is evident that though exact conversion of the constants of one equation to those of the other is not possible, the values obtained using Equations [18] are probably within experimental error. We include in Tables III, IV, and V the values obtained from Michels' data using Equations [18] except the 0° and 150° values which were obtained using Equation [2].

Baxter and Starkweather determined the densities of argon at 0°C. and pressures below 1 atm. The A_0 value calculated from their data by Cragoe (2)

TABLE III

COMPARISON OF A_0 VALUES FOR ARGON

	A_0, amagat atm.	S.E. of A_0, $\times 10^6$
This work	1.000999	10
Berlin	1.000984	
Berlin corrected	1.00096	
Amsterdam, equation [16]	1.00097	
Amsterdam, equation [21]	1.000963	7
Baxter and Starkweather	1.00100	

TABLE IV

COMPARISON OF 10^3B VALUES OF ARGON, AMAGAT/MOLE

T, °C.	Our values 10–80 atm.	Berlin 0–100 atm. (corrected)	Durham 5–125 atm.	Amsterdam 20–80 atm.
−100		−2.850		
− 50		−1.643		
0	−1.001	−0.96		−0.9578
25			−0.730	−0.703
50	−0.500	−0.465	−0.513	−0.502
75			−0.334	−0.324
100	−0.194	−0.167	−0.183	−0.179
125			−0.032	−0.053
150	+0.045	+0.088	+0.097	+0.062
174			+0.166	
200	+0.236	+0.223		
300	+0.481	+0.439		
400	+0.703	+0.669		
500	+0.793			
600	+0.870			

TABLE V

COMPARISON OF 10^6C VALUES OF ARGON, AMAGAT/ATM./MOLE

T, °C.	Our values 10–80 atm.	Berlin 0–100 atm. (corrected)	Durham 5–125 atm.	Amsterdam 20–80 atm.
−100		−10.2		
− 50		−0.84		
0	2.37	2.09		1.62
25			2.19	1.66
50	1.86	1.50	1.91	1.70
75			1.70	1.54
100	1.59	1.35	1.47	1.44
125			1.12	1.32
150	1.27	0.90	0.9	1.13
174			1.0	
200	0.96	1.03		
300	0.61	0.90		
400		0.2		

uses the results of only the first paper. We quote in Table III the mean of the results given in both papers.

ASSESSMENT OF BEST VALUES FOR B AND C

Since we do not expect our values of B and C to be very much more accurate than previous measurements, we have combined all the values obtained by various workers at the same temperature to obtain the best values of B and C. We based the weights to be assigned to the various values on the following considerations.

1. The Berlin, Amsterdam, Durham, and N.R.C. values have about the same standard error calculated from the internal consistency of the data.

2. The Berlin, Amsterdam, and Durham measurements include a volume and a mass measurement (measurement of P_0V_0 is equivalent to a mass measurement) and the temperature of the gas must be exactly at the required value or the measurements must be corrected to it since $A \propto T$. The N.R.C. measurements do not require a volume or a mass measurement and it is not so important that the temperature be exactly the temperature required on the thermodynamic scale. It is true that a volume ratio is needed, but this is obtained from the same measurements that give B and C so its inaccuracy is already included in the standard errors of B and C.

3. The Berlin data are subject to uncertainties for reasons outlined above.

4. Low pressure data are less accurate than high pressure data since the relative error in high pressure measurements is smaller than for low pressure measurements.

We have therefore given the following weights to the various measurements.

Baxter and Starkweather	1
Berlin	1
Durham	2
Amsterdam	2
N.R.C.	4

TABLE VI

WEIGHTED MEAN VALUES OF B AND C FOR ARGON

T, °C.	A	10^3B		Exptl. − calc.	10^6C		Exptl. − calc.	10^9D
		Exptl.	(Eq. 19)		Exptl.	(Eq. 20)		
−100	0.634539	−2.850	−2.846	−.004	−10.24			−13
− 50	0.817762	−1.643	−1.661	.018	− 0.84			22
0	1.000985	−0.987	−0.966	−.021	2.23	2.24	−.01	
25	1.092597	−0.717	−0.713	−.004	1.93	1.97	−.04	
50	1.184208	−0.499	−0.505	.006	1.80	1.75	.05	
75	1.275820	−0.329	−0.330	.001	1.62	1.57	.05	
100	1.367431	−0.185	−0.181	−.004	1.50	1.42	.08	
125	1.459043	−0.043	−0.053	.010	1.22	1.29	−.07	
150	1.550655	0.065	0.059	.006	1.12	1.17	−.05	
174	1.638602	0.166	0.153	.013	1.0	1.06	−.06	
200	1.733878	0.233	0.243	−.010	0.97	0.95	.02	
300	2.100324	0.481	0.503	−.022	0.61	0.58	.03	
400	2.466771	0.703	0.675	.028				
500	2.833217	0.793	0.794	−.001				
600	3.199663	0.870	0.878	−.008				

The Berlin 300°C. isotherm was rejected because of doubt about the temperature of the measurement (see above). Cragoe (2) points out that there is some doubt about the volume of the high pressure pipette as it shrunk by about 2 parts in 10^3 during a long series of experiments with argon and other gases. The Berlin 400° isotherm agrees rather badly with ours and the disagreement is of about the right order to be explained by this shrinkage. We have therefore not included the 400° isotherm in our mean values.

The weighted means of B and C are listed in Table VI. It is difficult to state an exact standard error for all the values of B and C since many of the measurements include appreciable constant errors. Standard errors of about 0.01×10^{-3} in B and 0.06×10^{-6} in C seem reasonable.

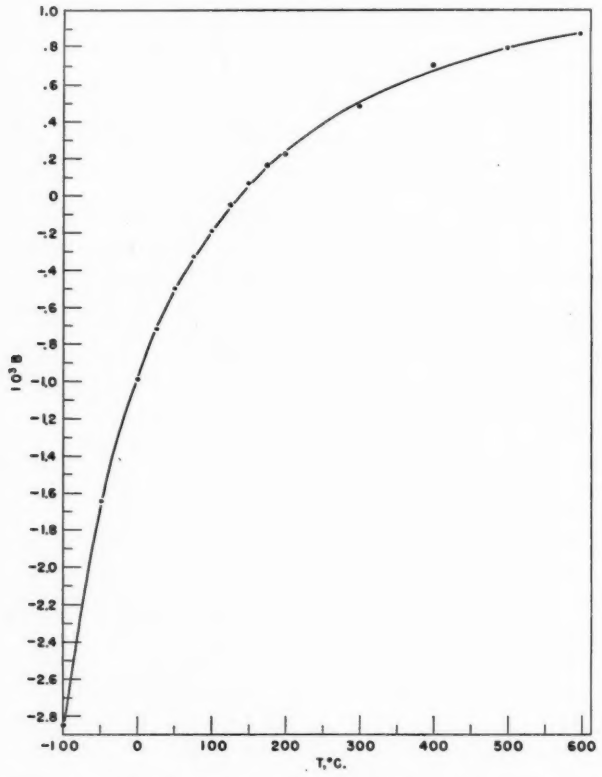

FIG. 1. B vs. T for argon. Values at -100 and $-50°$ C. are from Berlin; values from 0 to 200° C. are means; values from 200–600° C. are from this work. The line is Equation [19].

In Figs. 1 and 2 we plot the mean values of B and C respectively against temperature. The size of the dots indicates roughly the standard error. In Figs. 3 and 4 we compare the measured values of B and C obtained by various workers

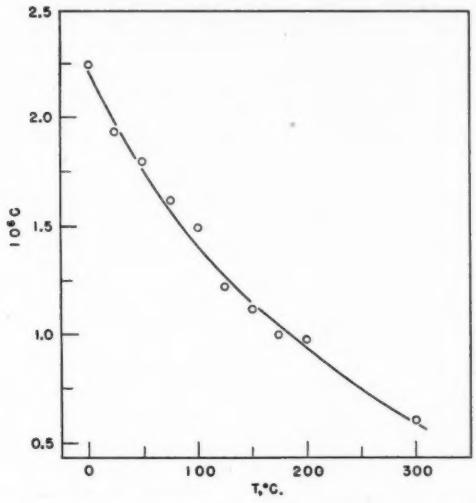

FIG. 2. C vs. T for argon. Values from 0 to 200° C. are means; the 300° C. value is from this work. The line is Equation [20].

with the mean value we have chosen. Outside the temperature range 0–200°C. no duplicate measurements have been included in the chosen values.

The normal volume using the averaged values of A_0 is 22,392.5 ± 2.2 cc./mole or 22.3919 ± .0022 l./mole.

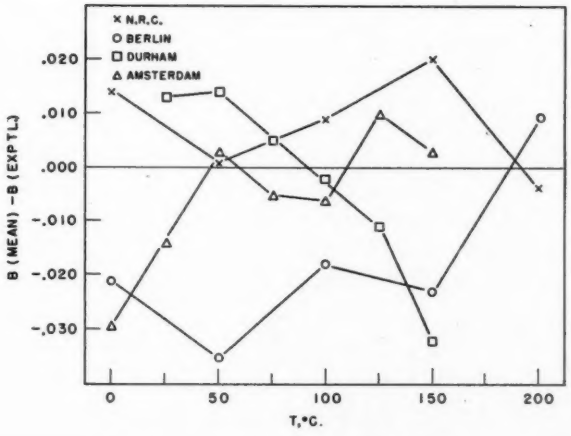

FIG. 3. B (mean) − B (exptl.) for argon.

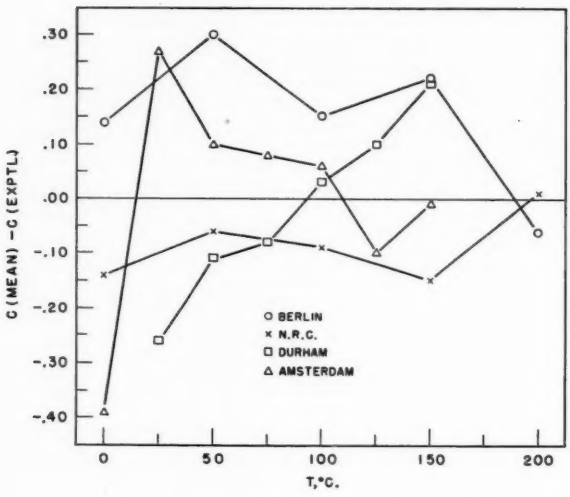

FIG. 4. C (mean) $-C$ (exptl.) for argon.

TEMPERATURE SERIES FOR B AND C

To facilitate interpolation of the virial coefficients and calculations of their derivatives with respect to temperature we have fitted the mean values of B and C to temperature series. Values of B over the range $-100°$ to $+600°$C. were fitted to the series

$$10^3B = a + bT + cT^{-1} + dT^{-3}$$

by the method of least mean squares, giving equal weight to all values. The following equation gave the best fit

[19] $$10^3B = 2.0358 - 3.140\times10^{-4}T - 769.34T^{-1} - 1.990\times10^6T^{-3},$$

with a standard deviation in 10^3B of 0.016. The experimental and calculated values using Equation [19] are given in Table VI. In Fig. 1 we compare directly the experimental values and Equation [19]. From Equation [19] and the deviation curve, the Boyle Point (temperature at which B is zero) is $138.2 \pm 2.3°$C.

Values of C over the temperature range 0–300°C. were fitted to the series

$$10^6C = a + bT + cT^{-3},$$

using the method of least mean squares and giving equal weight to all values. The following equation gave the best fit

[20] $$10^6C = 2.191 - 2.97\times10^{-3}T + 1.76\times10^7T^{-3}$$

with a standard deviation in 10^6C of 0.06. The experimental and calculated values are given in Table VI. In Fig. 2 we compare directly the experimental values and Equation [20].

ACKNOWLEDGMENTS

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THE pH DEPENDENCE OF THE ADSORPTION ISOTHERM AND ABSORPTION SPECTRUM OF METHYL ORANGE BOUND TO HUMAN AND BOVINE SERUM ALBUMIN[1]

By J. Ross Colvin

ABSTRACT

The difference between adsorption isotherms and absorption spectra of methyl orange bound to human and to bovine albumin at pH 6.8, 9.1, and 11.0 has been studied. Exaltation of the spectrum of methyl orange bound to human albumin is not necessarily correlated with total binding capacity. However, above pH 6.8, heterogeneity of the binding sites for methyl orange on human albumin increases so markedly that it is reflected in an appreciable increase in extinction coefficient for the first three or four anions bound. The exaltation is accompanied by an increased $-\Delta F^{\circ}$ of binding. Increase in ionic strength diminishes exaltation, and denaturation of the protein destroys it. These effects were not observed for bovine albumin. Results are interpreted in terms of a limited reversible expansion of the human protein molecules, without unfolding, due to intramolecular, electrostatic repulsion between groups.

INTRODUCTION

New techniques have recently encouraged the quantitative study of adsorption of ions by proteins in solution (6, 10, 12). These studies indicated that mechanisms of adsorption of organic anions by serum albumins from different species are similar (7). However, significant differences in total binding capacity for anions, and in mode of interaction, have been reported lately between samples of human and bovine serum albumin at pH's above 6.8 (8, 9). Studies reported here confirm and extend these observations.

Klotz, Burkhard, and Urquhart (8, 9) observed that as pH increased, total binding capacity of human and bovine albumin for methyl orange and related dyes increased unexpectedly but the increment was much greater for human albumin. In addition, there was an exaltation of the spectrum of dye anions bound to human albumin while the extinction coefficient of those bound to bovine albumin decreased slightly. Because such differences are important for a better understanding of the mechanism of adsorption reactions, these experiments have been repeated and extended to higher free anion concentrations. Relative heterogeneity of binding sites for methyl orange on human and bovine albumin has also been studied. Results confirm the previous observations on the spectra of methyl orange bound to the two proteins but also show that an exaltation of the spectrum of methyl orange bound to human albumin is not necessarily correlated with an increase of total binding capacity. Furthermore, exaltation of extinction coefficient of methyl orange bound to three or four sites on human albumin is accompanied by greatly increased heterogeneity among the remainder. An alternate interpretation of the original observations is suggested.

[1] *Manuscript received April 27, 1953.*
Contribution from Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3030.

METHODS AND MATERIALS

Adsorption isotherms were obtained by the method of dialysis equilibrium, which involves determination of the distribution of dye anions between a protein solution on one side of a semipermeable membrane and a buffer solution on the other. Techniques were as described previously (3). Vials were rotated overnight in a water bath which was held at the desired temperature ±0.1°C. Optical densities of the buffer solutions were measured at room temperature by a Beckman spectrophotometer Model DU at 5400Å. The protein concentration was about 0.2% for most experiments, corrected for moisture as determined by drying *in vacuo* at 100° for 16 hr. Molecular weight of all samples of albumin was assumed to be 69,000.

Absorption spectra of protein – methyl orange solutions were determined by a Cary recording spectrophotometer, Model 11M, Serial 95, over the range 3000–6000 Å. When only the absorption spectrum of the anion bound to protein was desired, a solution of the anion in the corresponding equilibrated buffer solution was used as blank. This procedure assumes that, as in dialysis equilibrium, free anion concentrations are equal in both solutions after equilibration.

Human and bovine albumin were denatured by heating solutions at the temperature of boiling water for 30 min. under reflux. At pH levels used in these experiments, both albumins remained in solution.

Intrinsic viscosities of protein solutions were estimated by the techniques outlined by Weissberger (13) using a capillary viscometer at 26.00 ± 0.05°C.

Specific optical rotations of protein solutions were determined with an A. Hilger Ltd. polarimeter, precision ±0.01°; final error of the specific angle was about ±0.5°. Tubes two decimeters long were used at room temperature with a sodium lamp as light source. The recorded estimate was the mean of 10 readings.

A commercial sample of methyl orange was purified by recrystallizing once from water, washing with alcohol, then ether, and subsequently drying the residue over calcium chloride. Extinction coefficient in 0.05 *M* phosphate, pH 6.8 at 4500 Å, 25°C., was 23,000 liters $mole^{-1}$ cm^{-1}.

Bovine serum albumin was purchased from Armour and Co., Chicago; Control No. 128–165.

Two samples of human serum albumin were employed. Sample I was prepared by Squibb and Sons, New Jersey, using method six of Cohn *et al.* (2) with the addition of cysteine monohydrochloride to remove mercury present as merthiolate. A 25% solution of this sample was then heated at 68°C. for 10 hr. It was subsequently reworked by fractionation with 10% alcohol and precipitation at pH 4.87 with 40% alcohol. Purity was greater than 99% by electrophoretic analysis. The small amount of impurity appeared to be an alpha globulin. Sample II was prepared by Cutter Laboratories, Calif., according to method six of Cohn *et al.* (2).

Buffers were made by Clark's procedure (1) from reagent grade materials.

RESULTS

Adsorption isotherms of methyl orange on human serum albumin (Sample I) and on bovine serum albumin were determined at 3° ± 0.1°C. over a wide

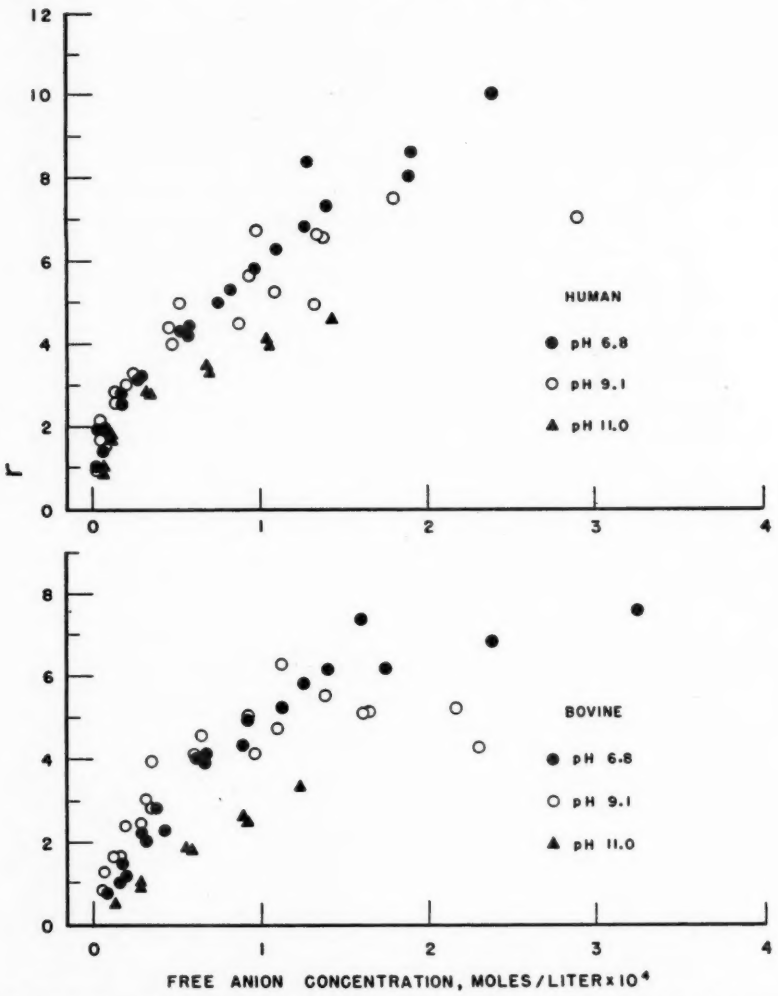

FIG. 1. Adsorption isotherms of methyl orange on human (Sample I) and bovine serum albumin in 0.05 *M* phosphate buffer pH 6.8, 0.05 *M* borate pH 9.1, and 0.07 *M* sodium hydroxide – phosphate buffer pH 11.0, at 3.0° C. The ordinate, *r*, is the average number of anions bound per protein molecule.

range of free dye concentrations for pH 6.8, 9.1, and 11.0 in 0.05 *M* phosphate, borate, and sodium hydroxide – phosphate buffers. Results are given in Fig. 1. Concentrations of free dye are plotted on a linear scale rather than logarithmic to illustrate experimental errors at high concentrations of dye. Contrary to the results of Klotz *et al.* (8, 9), there was no significant change in total binding capacity of either sample of albumin as pH increased from 6.8 to 9.1 but there was a decrease in total capacity for both proteins at pH 11.0. Lack of significant

change in total binding capacity for both albumins between pH 6.8 and 9.1 was confirmed by a second series at 30°C.

These observations were strengthened by a third series of experiments on Sample II of human albumin. Adsorption isotherms for methyl orange on this sample are shown in Fig. 2, as a function of pH. Clearly, these corroborate

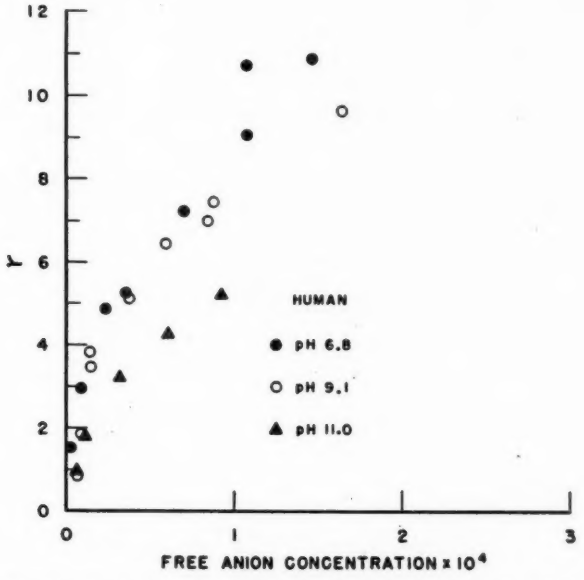

FIG. 2. Adsorption isotherms of methyl orange on human serum albumin (Sample II) in 0.05 *M* phosphate buffer pH 6.8, 0.05 *M* borate pH 9.1, and 0.07 *M* sodium hydroxide – phosphate buffer pH 11.0 at 3°.

the data of Fig. 1. Taken together, both figures show that adsorption capacity of human and bovine serum albumin for methyl orange does not necessarily increase with an increase in pH from 6.8 to 9.1 and that the capacity of both proteins decreases at pH 11.0.

Observations of Klotz *et al.* (8, 9) on the effect of human serum albumin on the spectrum of methyl orange at pH's above 6.8 have been completely confirmed, however. A progressive exaltation of the spectrum of adsorbed methyl orange was observed for both samples of human albumin as pH increased. Typical results are shown in Fig. 3. Although they extend over a wider range, these confirm results previously reported (8). Exaltation increases progressively from pH 6.8 to 9.1 and then remains constant from pH 9.1 to 11.0. This effect was not observed for bovine serum albumin and, as pointed out by Klotz *et al.*, indicates a marked difference in the interaction of organic anions with these two proteins.

Exaltation of the spectrum of methyl orange bound to human serum albumin is dependent on a specific structure of the adsorbent, for heat denaturation

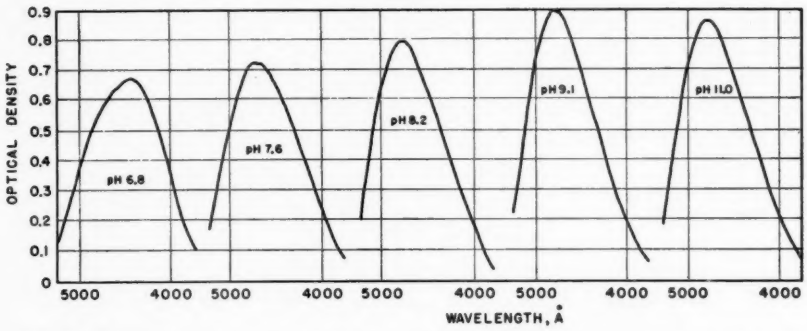

FIG. 3. Absorption spectra of methyl orange – human serum albumin mixtures at increasing pH levels. Concentration of methyl orange in all solutions 0.3×10^{-4} M; protein concentration of 0.2%.

completely abolished the effect. Although not unexpected, this conclusion could not be assumed since the increase in extinction coefficient might well be due to interaction with stable groups which become more easily available either on denaturation or as pH increased.

The relation between optical density of a solution of bound anions of constant protein concentration and fixed light path, and the number of anions bound per protein molecule may give information about homogeneity of binding sites. For instance, if all anions are bound by identical mechanisms, there will be a direct proportionality between optical density of such a solution and number of anions bound. On the other hand, variations in manner of binding over the range of adsorption may lead to departure from linearity in this relation provided that the protein–anion interaction is strong enough to perturb the light adsorbing portions of the dye. It is impossible, except in special cases, to obtain a solution containing only bound dye anions. However, using the Cary recording spectrophotometer with an equilibrated free anion solution as blank the optical density of such a hypothetical solution may be estimated accurately and plotted against the independently determined r value. This has been done for both bovine and human serum albumin at pH 6.8, 9.1, and 11.0 over the widest practicable concentration range. Results are shown in Figs. 4 and 5. Visual inspection shows that the relation between optical density of bound methyl orange solution and r is linear over the whole concentration range, for both bovine and human serum albumin at pH 6.8. Statistical analysis confirms this (Table I). Therefore, within the limits of this insensitive test, all binding sites of methyl orange on both human and bovine albumin are equal at pH 6.8. This is also true for bovine albumin at pH 9.1 and 11.0, although the data are less reliable at pH 11.0. However, the relation between optical density of bound methyl orange solution and r is curvilinear for human albumin at pH 9.1 and 11.0 (Table I). Inspection of the curves shows that for human albumin at both pH's, average optical density per dye anion bound is greater for the first three anions adsorbed than that for all anions bound at pH 6.8. The average extinction coefficient per anion bound declines at both pH levels as total number bound

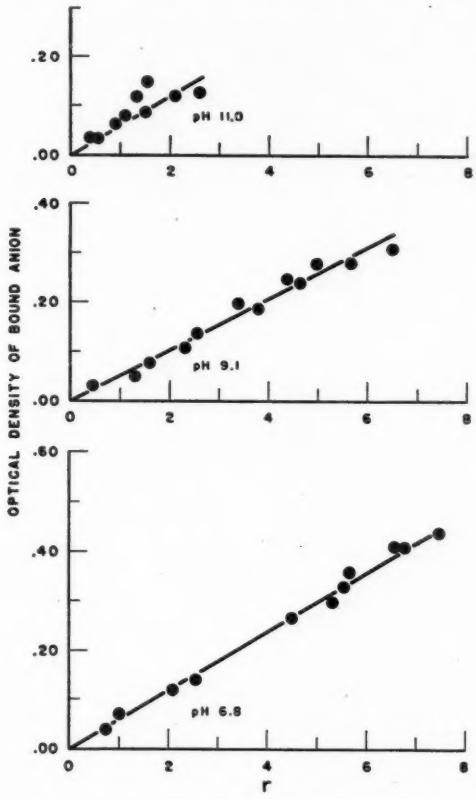

FIG. 4. Relation between optical density of a solution of methyl orange anions bound to bovine serum albumin and r, the average number of bound anions per protein molecule, at pH 6.8, 9.1, and 11.0. Protein concentration constant at 0.2%; light path constant at 1 mm.

TABLE I

TEST OF LINEARITY OF RELATION BETWEEN D, OPTICAL DENSITY OF SOLUTIONS OF METHYL ORANGE BOUND TO HUMAN OR BOVINE ALBUMIN, AND r, AVERAGE NUMBER OF ANIONS BOUND PER PROTEIN MOLECULE, AT pH 6.8, 9.1, AND 11.0; PROTEIN CONCENTRATION 0.2%, LIGHT PATH 1 MM.

System	pH	No. of deter.	Mean r	Mean D	Slope of D versus r with 95% confidence limits	Ordinal intercept with 95% confidence limits
Bovine – methyl orange	6.8	11	4.37	0.26	0.061 ± 0.004	-0.004 ± 0.009
	9.1	12	3.46	0.180	0.051 ± 0.006	0.003 ± 0.012
	11.0	10	1.23	0.083	0.055 ± 0.028	0.016 ± 0.020
Human – methyl orange	6.8	12	5.72	0.352	0.061 ± 0.002	0.003 ± 0.005
	9.1	12	4.11	0.244	0.050 ± 0.005	0.037 ± 0.010
	11.0	12	2.83	0.167	0.039 ± 0.008	0.056 ± 0.010

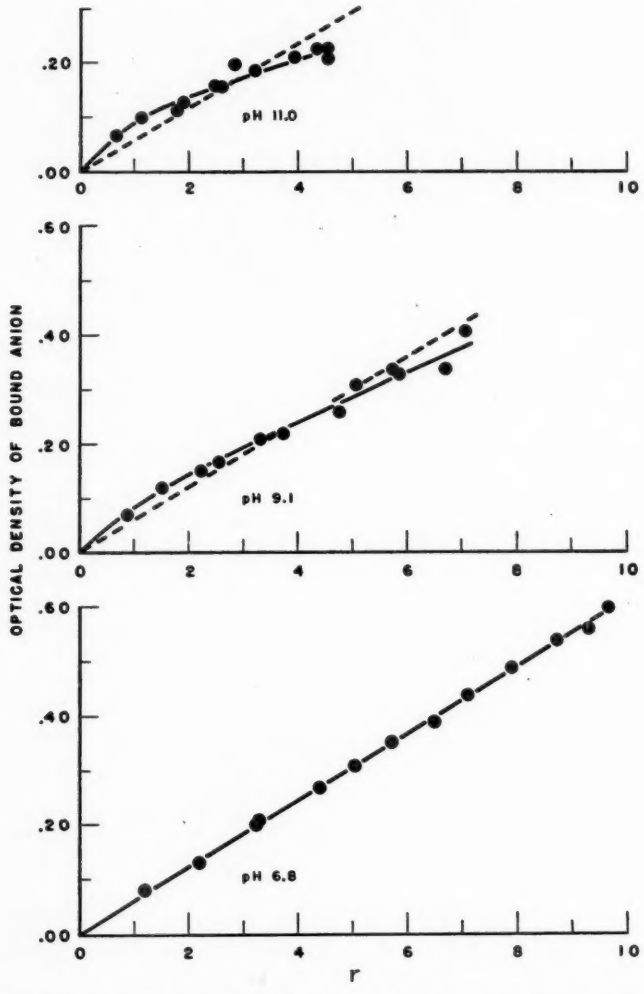

FIG. 5. Relation between optical density of a solution of methyl orange anions bound to human serum albumin and r, the average number of bound anions per protein molecule, at pH 6.8, 9.1, and 11.0. Protein concentration constant at 0.2%; light path constant at 1 mm. The dotted line at pH 9.1 and pH 11.0 indicates the relation at pH 6.8.

increases, showing that methyl orange anions must be bound very unequally to human albumin at each of these pH's. Furthermore, considering that the average extinction coefficient of anions bound to bovine albumin decreases as pH increases (Table I), the first three or four anions must be bound with an abnormally large free energy decrease in such a way that they cause an exaltation of the spectrum of bound methyl orange and the remainder cause a decrease.

In contrast, as shown above, anions are bound relatively equally to bovine serum albumin at all dye concentrations for each pH.

TABLE II

WAVE LENGTHS OF MAXIMUM ABSORPTION OF METHYL ORANGE BOUND TO HUMAN AND BOVINE PLASMA ALBUMIN AS A FUNCTION OF r, NUMBER OF ANIONS BOUND PER PROTEIN MOLECULE, AT pH 6.8, 9.1, AND 11.0

6.8		9.1		11.0	
r	λ_{max}, Å	r	λ_{max}, Å	r	λ_{max}, Å
Human					
1.2	—	0.9	4850	0.7	4800
3.3	4400	2.3	4700	1.8	4750
4.4	4400	3.7	4600	2.6	4650
7.1	4350	5.0	4400	3.2	4500
8.8	4400	5.7	4350	3.9	4350
9.7	4350	7.0	4350	4.4	4300
Bovine					
0.8	4500	0.5	4400	0	4400
2.1	4400	1.6	4500	0.4	4400
—	—	2.6	4350	0.9	4250
5.3	4300	3.4	4350	1.5	4300
5.7	4250	4.4	4300	2.1	4250
6.6	4300	5.0	4300	2.6	4200

These conclusions were confirmed by shifts in the absorption maxima of the spectra of the bound methyl orange complexes (Table II). At pH 6.8, wave length of maximum adsorption of methyl orange bound to both human and bovine albumin was nearly independent of average number of anions bound per protein molecule. This was also true of complexes with bovine albumin at pH 9.1 and 11.0. For complexes of methyl orange with human albumin at both pH 9.1 and 11.0, however, λ_{max} shifted steadily toward shorter wave lengths as r increased.

Increasing the ionic strength should tend to eliminate the difference in absorption spectra between the two albumins because of the competitive binding of other anions, decrease in internal electrostatic repulsion, and decrease in Donnan pressure (11). Accordingly, the spectrum of methyl orange bound to both samples of human albumin was determined at pH 9.1 and 11.0 in concentrations of sodium chloride varying from zero to 2.0 *M*. Typical spectra are shown in Fig. 6. As expected, optical density of the solution decreases with increasing ionic strength but the rate of decrease is surprisingly small. Clearly, those sites responsible for the exaltation of the spectrum of bound methyl orange are little affected by the presence of ions in the external solution. This is consistent with the abnormally large free energy decrease associated with binding to these sites.

If exaltation of extinction coefficient of methyl orange bound to human plasma albumin were attendant on a major configurational change of the

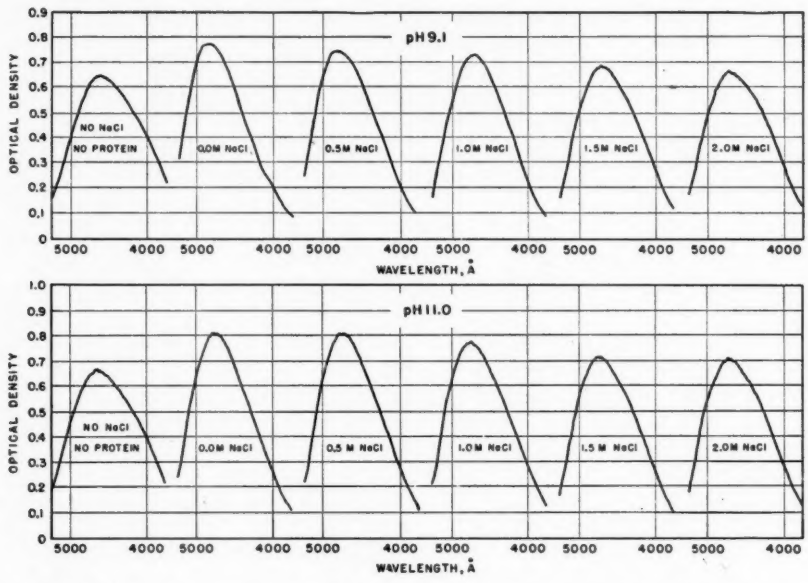

FIG. 6. Absorption spectra of methyl orange – human serum albumin mixtures for increasing sodium chloride concentrations at pH 9.1 and pH 11.0. Methyl orange concentration 0.3×10^{-4} M; protein concentration 0.2%.

protein, such an alteration might be detectable by a change in intrinsic viscosity or specific optical rotation as pH increased. Since intrinsic viscosity is equal to $(\eta_{sp}/c)_{c \to 0}$(13), such a change with pH should be reflected in a change of slope of a plot of specific viscosity against concentration. Fig. 7 summarizes results of a determination of specific viscosity of human and bovine albumin at pH 6.8 and 9.1 as a function of concentration. Statistical analysis shows that the slopes of the functions, and therefore intrinsic viscosities, are equal at pH 6.8 and 9.1, for both proteins. Likewise, determination of specific optical rotation of both human and bovine albumin in duplicate at pH 5.5, 6.8, and 9.1 yielded values varying from $-55°$ to $-62°$ in agreement with previous work (5) but no significant differences could be observed between the two proteins in the manner in which this parameter varied with pH. Consequently, both types of evidence indicated that any configurational change is minor. This is consistent with the reversibility of the effect (8).

DISCUSSION

The isotherms of Figs. 1 and 2, together with spectra of Fig. 3, show that there is no general positive correlation of increase in binding capacity of human albumin with increase in extinction coefficient of bound anion as pH increases. On the contrary, as would be expected from electrostatics, total binding capacity decreases while exaltation increases with increasing pH, up to a limit. Furthermore, Fig. 5 shows that after three anions are bound to human albumin at pH

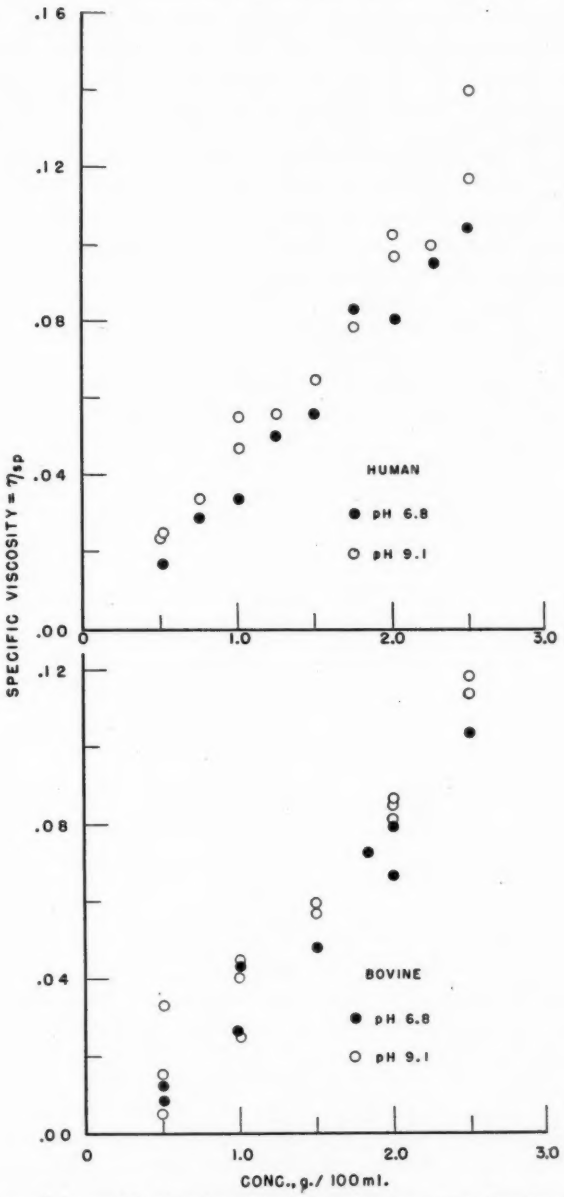

FIG. 7. Plots of the specific viscosity of bovine and human serum albumin against concentration in gm./100 ml. for pH 6.8 and 9.1.

Probability, P, that the difference between the intrinsic viscosities of human albumin at pH 6.8 and pH 9.1 would be obtained by chance is 0.2; for bovine albumin between pH 6.8 and 9.1, $P = 0.3$; and for the difference between bovine and human at pH 6.8, $P = 0.7$.

9.1 or 11.0, the average extinction coefficient per anion bound is progressively less than that of anions bound at pH 6.8. Therefore, only the first few anions bound are responsible for exaltation of the spectrum of the complex. The decrease in average extinction coefficient for anions bound to bovine albumin, as pH increases (Table I), strengthens this conclusion. Consequently, unless all binding sites at pH 6.8 are different from those at pH 9.1, the exaltation is probably not caused by the binding of anions to new sites made available by unfolding of the molecule but by anions bound to a few formerly equivalent sites which have been strongly modified by expansion of the molecule under Coulomb forces (9). In fact, these results can be interpreted as direct evidence for the type of expansion envisaged by Scatchard (11). The heterogeneity of binding shows that this expansion permits suitable groups such as the tyrosine side chain (9) to perturb the light absorbing system of the anions in perceptibly different ways. However, only three or four of these anions are influenced in such a way that their molecular extinction coefficient is increased. Because these anions tend to be bound first, their free energy decrease must be appreciably greater than for remaining positions, in addition to the statistical effect (7).

The strong binding of the first anions to human albumin at pH's above 6.8 is probably responsible for the apparent discrepancy between results of Klotz, Burkhard, and Urquhart (9) and those reported here. Klotz *et al.* determined adsorption isotherms for methyl orange on both human and bovine albumin over a range of r values extending only from zero to four. Consequently, they concluded that exaltation of the spectrum of methyl orange bound to human albumin was generally accompanied by an increase in total binding capacity. Results reported here, which do not confirm this correlation, were foreshadowed by the fact that although their sample 179 − 5X had the greatest adsorption affinity for methyl orange, a second sample from Cyanamid had the largest effect on the spectrum of the anion at pH 7.6.

A similar explanation probably also applies to lack of decrease of exaltation at pH 11.0. In Fig. 4 exaltation increases with pH from 6.8 to 9.1 and then remains constant, although total binding capacity decreases markedly. Those bound anions which are chiefly responsible for exaltation are therefore least affected by increased electrostatic repulsion. This would be expected if they are those bound most strongly. Lack of effect of pH on these sites also suggests that expansion of the molecule must be strictly limited by some strong covalent bonds. Otherwise the suitable spatial relation of given sites would be destroyed by continual expansion and therefore exaltation should decrease. However, since it is not known whether sites occupied first at pH 9.1 are the same as those initially occupied at pH 11.0, this argument cannot be conclusive.

Postulation of such strong covalent bonds also offers a reasonable explanation of the difference between human and bovine albumin at pH's above 6.8. Apparently, for bovine plasma albumin, these covalent links may be strong enough to completely prevent any appreciable modification of binding sites for methyl orange at all pH's. In addition, human albumin may contain three or four more suitably situated hydroxyls, relative to cationic sites. Further investigation is needed to test these possibilities.

These results appear to provide a direct confirmation of the common assumption that binding sites for methyl orange on bovine serum albumin at pH 6.8 are equivalent (7), in apparent refutation of previous criticisms of the validity of this postulate (3, 4). However, the insensitive test for homogeneity applied in this study could not show effects even of major variations in local topography which did not directly perturb the light absorbing system of the anion. Linearity between optical density of a solution of bound anions and average number of anions bound per protein molecule is a necessary but not a sufficient condition for equality of sites.

ACKNOWLEDGMENTS

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THE SENSITIVITY OF INITIATOR EXPLOSIVES TO MECHANICAL IMPACT[1]

By N. R. S. Hollies,[2] N. R. Legge,[3] and John L. Morrison

ABSTRACT

An original ball-drop impact apparatus has been constructed and used to test the sensitivities of dextrin lead azide and mercury fulminate to mechanical impact. Ball masses and heights were both varied. Both the net kinetic energy and the change of momentum gave continuous functions with the percentage of detonations. Comparisons of the absolute values of the net kinetic energy and the change of momentum with another reported in the literature suggest that momentum is the more important factor in determining the probability of detonation. Values for the momentum at 50% detonations for lead azide and mercury fulminate are 4.6×10^4 and 2.6×10^4 c.g.s. units, respectively, with an area of contact of about 0.08 cm.^{2} The corresponding times of impact were found to be about 2.2×10^{-4} and 1.9×10^{-4} sec.

The question of whether the probability of the detonation of an initiator explosive is chiefly determined by the kinetic energy or the momentum of the impacting mass is of theoretical interest in interpreting the process of detonation by impact (8). In their earlier work, Taylor and Weale (9) suggest that kinetic energy is the important factor, although in 1938 (10) they introduce experiments and calculations based on momentum. Taylor and Weale do not mention the rebound of the steel balls used in their impact tests, although the measurement of rebound was introduced decades ago (referred to in Ref. 11). Powell, Skelly, and Ubbelohde (8) measured the momenta and kinetic energies of impact for mercury fulminate and lead styphnate. In their calculations, they assumed without measurement a definite value for the coefficient of restitution of the impacting mass. In the present work, some measurements of the net kinetic energy, change of momentum (hereinafter called impulse), and time of impact for the initiator explosives mercury fulminate and lead azide are reported. The time measurements allowed estimations to be made of the forces involved and these were found to be comparable with those found by Taylor and Weale (10) by an entirely independent method.

EXPERIMENTAL

Materials

Dextrin lead azide was prepared from sodium azide which had been recrystallized from a water–acetone mixture. Separate 50 ml. aqueous solutions containing 4 gm. sodium azide and 10.2 gm. lead nitrate C.P. were simultaneously added from burettes (delivery time—134 sec.) into 250 ml. of 0.5% aqueous solution of dextrin. Microscopic examination showed that the crystals of lead azide were of the same form as those prepared by Lowndes (6).

[1] *Manuscript received February 24, 1953.*
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Mercury fulminate was prepared by a method given by Davis (5, p. 406).

Impact Apparatus

The essential parts of the impact apparatus are shown in Fig. 1. The hammer

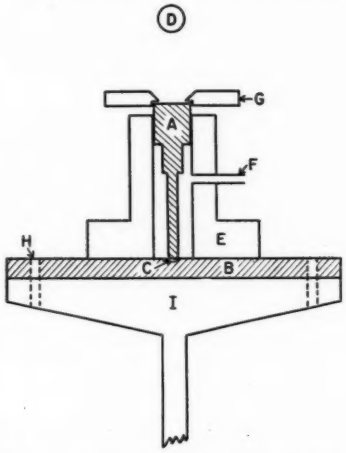

FIG. 1. Impact apparatus (schematic).

A—hammer	D—steel ball	G—steel ring for static load
B—base plate	E—hardwood guide	H—bolt hole
C—cavity in base plate	F—vent to suction	I—I beam

was made from the rear axle of a 1929 model Pontiac automobile. It rested in a concentric cavity in a 0.5 in. thick steel base plate which had been cut from a high carbon steel road grader blade. The steel base plate was carefully faced and fastened securely by six large bolts to the smoothed upper face of an 18 in. I beam (approx. wt. = 110 lb.). Both the hammer and base plate were hardened by heat treatment and quenching. This arrangement of base plate and I beam ensured a large mass *as well as* the possibility of hardening the anvil. In all the experiments the hammer was under a static load obtained by means of a second class lever. The total load, including the hammer of 211 gm., was 40,098 gm.

Steel balls (S.K.F. ball bearings) of various sizes were dropped on the hammer by release from an electromagnet and their rebound was measured by the shadow cast on a lighted calibrated scale.

The original cavity in the base was obtained by bouncing a $1\frac{1}{2}$ in. diameter ball (larger than any used in the impact tests) on the hammer. Then, in carrying out a series of tests, the smallest balls and heights of fall were used at first, and these were increased in succession. This procedure prolonged the good condition of the base plate cavity and of the hammer. Whenever the end of the hammer became pitted by explosions, it was reshaped on a lathe to a radius of curvature of 5/32 in. with successive emery paper sizes 2, 00, and 0000. This procedure was necessary after 15 to 20 detonations of lead azide. Then the cavity in the base plate was repounded until the normal bounce height was secured.

In carrying out an impact test, the cavity in the base plate was filled with initiator explosive by dusting with a camel's hair brush. (The initiator was kept over calcium chloride between tests.) The hammer and weight arm were gently lowered on top of the sample and the extra weight was then placed on the weight arm.

There was no difficulty in detecting the occurrence of an explosion, although mercury fulminate exhibited the phenomenon of partials a few times.

Time of Impact Measurements

The time of impact was measured in a way similar to that used by Bowden and Tabor (4). A wave controller using a cathode ray oscilloscope was designed and built by Dr. H. E. Johns* and one of us (N.R.S.H.). The controller used a thyratron which was applied to the horizontal plates to produce a linear beam of definite periodicity across the oscilloscope screen. At the same time a transient, which was produced by the closing of a voltage circuit by the impact of the hammer on the base plate, was applied to the vertical plates. The resulting impact wave trace on the oscilloscope screen was photographed with Kodak Verichrome film. The period of the trace was calibrated by photographing the trace of a standard 10,000 or 20,000 cycle sec.$^{-1}$ wave on the same film.

Because of a poor estimate of the expected length of the wave trace, the firing mechanism was inaccurately timed, and the length of the transient was estimated to be as much as 25% too short; that is, the times listed in Table III are at the most about 25% low; however, they are still related relatively.

Bases of the Calculations

The percentage of detonations are compared with (*a*) the kinetic energy assuming no rebound of the ball, (*b*) the net kinetic energy, and (*c*) the impulse or change in momentum. These are given by the following equations, respectively:

$$(a)\ mgh,$$
$$(b)\ mg(h_1 - h_2) = 980\, mh_1(1 - e^2),$$
$$(c)\ Ft = m(v_1 - v_2) = 44.3\, m(1 + e)h_1^{\frac{1}{2}},$$

where m = ball mass, g = gravitational constant, h_1 = initial fall height, h_2 = rebound height, $e = v_2/v_1 = (h_2/h_1)^{\frac{1}{2}}$ = coefficient of restitution, F = force, t = time over which ball goes from v_1 to v_2, v_1 = falling velocity before duration of contact, v_2 = rebounding velocity after duration of contact.

RESULTS AND CALCULATIONS

Impact Tests

The results of about 500 impact tests on dextrin lead azide and 300 on mercury fulminate are given in Tables I and II respectively. At least 20 and sometimes 100 trials were made for each ball at each height (the numbers are given in brackets after the percentage detonations). In Figs. 2 and 3 the percentage detonations are plotted against gross kinetic energy, net kinetic energy, and impulse respectively.

Time of Impact

The times of impact as measured under various conditions are given in

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TABLE I

SENSITIVITY OF LEAD AZIDE TO KINETIC ENERGY AND IMPULSE

Ball diam., in.	Ball mass, gm.	Height (h_1), cm.	e	Detonations, %	mgh, ergs × 10^{-6}	Net kinetic energy, ergs × 10^{-6}	Impulse, c.g.s. units × 10^{-4}
7/8	44.66	90	0.645	10.0(20)	3.94	2.30	3.10
"	"	100	0.644	15.0(20)	4.37	2.56	3.26
"	"	110	0.643	17.5(40)	4.82	2.81	3.42
"	"	120	0.647	20.0(20)	5.25	3.07	3.58
1	66.68	60	0.510	17.5(40)	3.92	2.90	3.46
"	"	80	0.513	35.0(40)	5.23	3.86	4.03
"	"	100	0.514	47.5(78)	6.54	4.83	4.51
"	"	120	0.509	57.0(100)	7.84	5.79	4.93
1-1/8	95.04	100	0.372	70.0(20)	9.33	8.04	5.80
"	"	120	0.368	87.5(40)	11.20	9.65	6.32

TABLE II

SENSITIVITY OF MERCURY FULMINATE TO KINETIC ENERGY AND IMPULSE

Ball diam., in.	Ball mass, gm.	Height (h_1), cm.	e	Detonations, %	mgh, ergs × 10^{-6}	Net kinetic energy, ergs × 10^{-6}	Impulse, c.g.s. units × 10^{-4}
3/4	28.14	60	0.738	15.0(60)	1.65	0.75	1.67
"	"	90	0.738	31.7(60)	2.48	1.13	2.06
"	"	120	0.739	43.3(30)	3.30	1.50	2.38
7/8	44.66	60	0.636	45.0(60)	2.62	1.58	2.50
"	"	80	0.627	63.3(60)	3.50	2.12	2.89
"	"	100	0.628	85.0(60)	4.38	2.65	3.23

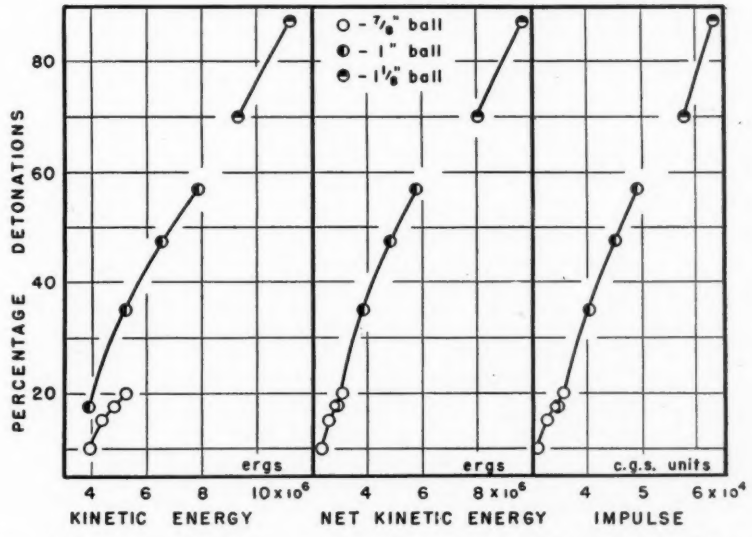

FIG. 2. Comparisons of the percentage of detonations of dextrin lead azide with gross and net kinetic energies and impulse.

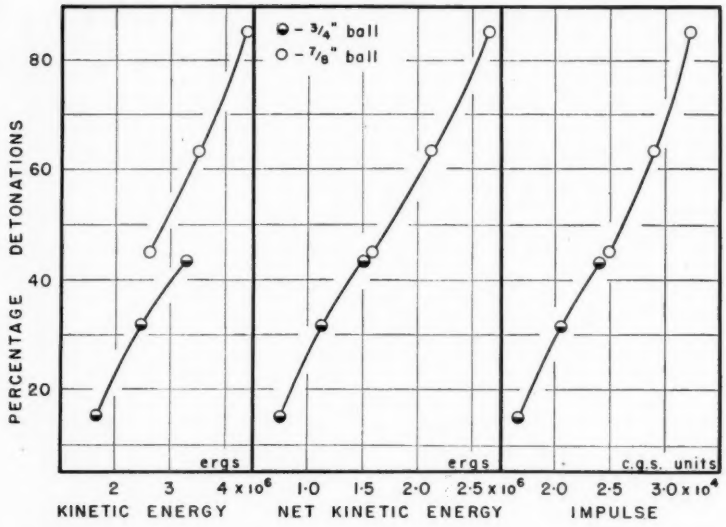

FIG. 3. Comparisons of the percentage of detonations of mercury fulminate with gross and net kinetic energies and impulse.

Table III. Between the tests on lead azide and mercury fulminate the base plate was reground and rehardened. This accounts for the slight change in the coefficient of restitution e of the 7/8 in. ball in Tables I and II. Also, in some of the time experiments, norite was placed in the cavity between the hammer and base plate. The use of norite probably simulated the conditions of impact when initiator crystals rested between the hammer and base.

Hertz's theory of colliding metal pieces (1) shows that $t(rv/m^2)^{1/5} = k$, a constant, where t = time of impact, r = radius of steel ball, $v = v_1$ = velocity before impact, m = ball mass. The constant k has been calculated for the norite time experiments and is included in Table III. The evaluation of k permits an estimation to be made of the time of impact within the lead azide detonation range where norite experiments were not made.

Calculation of Impulsive Pressures

Assuming that impulse determines the probability of detonation, an estimate of the impulsive pressure or force per unit area at 50% detonations can be made. The apparent area of contact between the hammer and base plate was 0.10 sq. cm. The hammer was under a static load of 400 kgm. cm.$^{-2}$ Under this compression, the 'true' area of contact with the initiators can be calculated from their crystal densities at known compressions (7). These are 0.88 of the apparent area for mercury fulminate, and 0.75 for lead azide.

For Mercury Fulminate

Total impulse at 50% detonations = 2.6×10^4 c.g.s. units. Time of impact at 50% detonations = 1.9×10^{-4} sec. Force = $I/t = 1.37 \times 10^8$ dynes. Impulsive

TABLE III

TIMES OF IMPACT

Ball diam., in.	Height (h_1), cm.	Time of impact, sec. $\times 10^4$	
Original base plate			
7/8	90	0.62	
"	100	0.72	
"	110	0.88	
"	120	0.94	
1	60	1.01	
"	80	1.28	
"	100	1.38	
"	120	1.48	
1–1/8	100	1.65	
Reground base plate			
3/4	90	0.21	
"	120	0.39	
7/8	60	0.39	
"	90	0.54	
"	120	1.08	
1	60	0.72	
"	90	0.83	
"	120	1.07	
Reground base plate with norite			k (Hertz) $\times 10^4$
3/4	60	1.49	1.25
"	90	1.63	1.43
"	120	1.81	1.59
7/8	60	1.83	1.31
"	90	2.01	1.49
"	120	2.16	1.63

pressure = $1.37 \times 10^8/0.88 \times 0.1 = 1.56 \times 10^9$ dyne cm.$^{-2}$ or 11.3 ton in.$^{-2}$

For Lead Azide

Total impulse at 50% detonations = 4.6×10^4 c.g.s. units. Time of impact at 50% detonations (based on norite times and Hertz's formula) = 2.2×10^{-4} sec. Impulsive pressure = $2.1 \times 10^8/0.75 \times 0.1 = 2.8 \times 10^9$ dyne cm.$^{-2}$ or 20.3 ton in.$^{-2}$

(Note: As the time of impact may be about 25% low, the above impulsive pressures may be about 25% high.)

Coefficient of Restitution

Values of the coefficient of restitution e are included in Tables I and II. Their measurement is a useful method of testing the stability of the impact apparatus. The constancy of e for any one ball over the range of fall heights used is considered an indication of a rigid system (1). The values of e were found to be completely independent of the presence or absence of initiator explosive crystals, or of the occurrence or nonoccurrence of detonations.

The relation between ball weights and e is given in Fig. 4. Shown are the results for (*a*) an earlier, more complex, apparatus which included a ball bearing

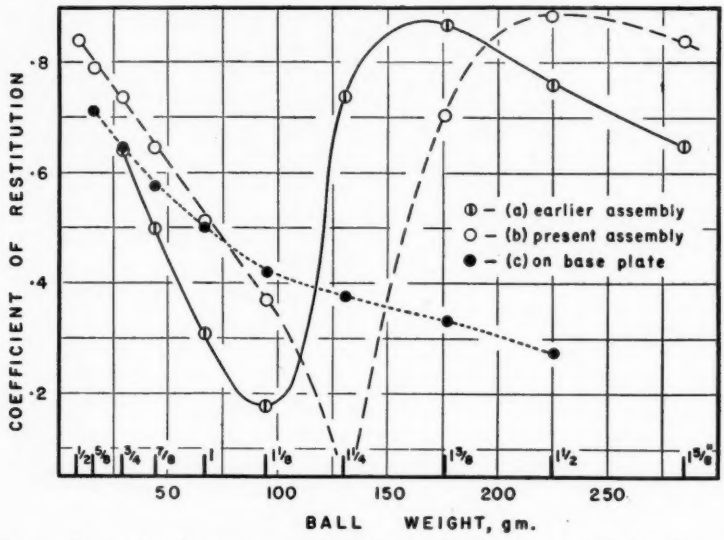

FIG. 4. The relationship between coefficient of restitution and ball weight for various assemblies.

as part of the hammer assembly, (*b*) the present apparatus (Fig. 1), and (*c*) the same balls when dropped directly on the base plate. It would appear that the curious periodic relation between ball weight and *e* arises largely from the hammer part of the apparatus, and may be related to its resonating frequency.

DISCUSSION

Although the range of fall heights used in the present work was not sufficient to give overlapping in the critical regions of percentage of detonations (Figs. 2 and 3), yet the general trend is quite clear. It is apparent that both the *net* kinetic energy and the impulse are continuous functions of the percentage of detonations. Thus, no discrimination between the relative importance of kinetic energy and impulse in determining the probability of detonation can be made merely by varying the size of balls. If this is true for all impact machines, then one must look in other directions for such a discrimination.

Kinetic energy is dissipated in many directions, whereas impulse is probably conserved in one direction. A comparison of the absolute magnitudes of the net kinetic energy and the impulse for different impact assemblies may provide the basis for a critical test of which is the more important factor in detonation. In the present case, the assembly could have been varied by altering the mass of the hammer—this was not attempted.

Powell, Skelly, and Ubbelohde (8) have also determined the variation of the percentage of detonations with kinetic energy and impulse for mercury fulminate. Their value for the net kinetic energy at 50% detonations was about 8×10^3 ergs, compared with the present result of 1.7×10^6 ergs. On the other hand, their value for the impulse at 50% detonations was 1.2×10^4

c.g.s. units, which is quite comparable with the present value of 2.6×10^4 c.g.s. units on the following grounds. Their area of contact appears to be about *one half* of the present one; their drift, containing a 3/16 in. diameter ball bearing, rested on a flat brass disk which was resting in turn on a prepressed sample of the initiator explosive; in the present case, the end of the hammer is the equivalent of a 5/16 in. diameter ball, and it rests on a sample of the initiator which is resting in turn in a cavity concentric with the end of the hammer. Thus, it would appear on the basis of the above comparisons involving radically different impact machines that impulse is the more important factor in initiating detonation.

In their calculations, Powell *et al.* assumed a value of 0.9 for the coefficient of restitution. Moreover, their impact assembly was not rigid: "a fraction of the momentum of the drift is lost owing to imperfect rigidity of the supports for the initiator (i.e. the brass disks, the Hoffman roller, etc.)"—(8, p. 294). It is possible that their results would have been more consistent if they had measured *e* and used a more rigid assembly. In spite of this, the impulses for two different drifts are parallel to each other over the range of percentage of detonations, and only differ by about 20% in the case of mercury fulminate and 12% in the case of lead styphnate.

A hypothesis which favors kinetic energy is that of Bowden and Gurton (2) who, on the basis of work with added grits, come to the conclusion that the initiation of explosives by mechanical impact is a frictional phenomenon. In their words: "The mechanical energy of the blow must first be degraded to heat to form a hot spot of small but finite size. Thermal initiation then occurs at this hot spot." This round-about transfer of energy (i.e. a transfer from mechanical to thermal to chemical energies) is difficult to conceive on thermodynamic grounds. The more direct transfer from mechanical to chemical energies would be suggested if impulse is the factor determining the probability of detonation on impact.

In another paper (3), Bowden and Gurton report what they designate as "the delay between impact and explosion" *times* measured by means of the high speed photography of actual explosions on impact. Practical difficulties prevented the measurement of the times for ordinary lead azide and mercury fulminate but in the presence of a glass grit particle, they were of the order of 1×10^{-4} sec. This is very similar to the times of impact reported in the present work, and would suggest that the so-called 'delay'—presumably to allow mechanical energy to be changed to thermal energy and then to detonation—may be none other than the normal time of impact connected with the direct transfer of momentum. For larger crystals of lead azide, Bowden and Gurton found the 'delay' time to be about 1×10^{-5} sec., an observation difficult to account for on the basis of their hypothesis, but not so on the basis of the momentum hypothesis.

The magnitude of the impulsive pressures necessary for the detonation of mercury fulminate may be compared with another estimate available in the literature. Taylor and Weale (10) compared the impulse necessary to detonate mercury fulminate with that necessary to indent a lead block. They concluded

that "the impulsive stress required to initiate a thin layer of mercury fulminate was not less than 7 tons per sq. in." (10, p. 999). Further, they estimated the time of impact to be of the order of 10^{-5} sec.

Some realization of the apparatus difficulties involved in this type of work may be seen by an examination of the velocities of impact necessary for the detonation of initiator explosives. Referring to the work of Andrews (1) and of Bowden and Tabor (4), it is apparent that the velocities used here (343 to 486 cm. sec.$^{-1}$) are well beyond the velocity where the plastic deformation of steel sets in. Bowden and Tabor found that for very hard tool steel at higher velocities (100 to 200 cm. sec.$^{-1}$) the conductance curves for the time of impact became asymmetric, showing that plastic deformation occurs. Andrews has shown that Hertz's well-known formula only strictly applies for pure elastic rebound, which only occurs at low velocities of the order of a few cm. sec.$^{-1}$ It would appear, however, from the present results, that the occurrence of some plastic deformation is not too important provided that an otherwise *rigid* assembly is used in impact tests.

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STUDIES OF LIGNIN BIOSYNTHESIS USING ISOTOPIC CARBON

II. SHORT-TERM EXPERIMENTS WITH $C^{14}O_2$[1]

By Stewart A. Brown, K. G. Tanner, and J. E. Stone[2]

ABSTRACT

The formation of lignin in wheat plants from $C^{14}O_2$, during the period of rapid lignification, has been studied over the first few hours of photosynthesis by measuring the incorporation of carbon-14 into the guaiacyl and syringyl portions of the lignin molecule. Plants grown 62 days from seeding were exposed to 20 microcuries of $C^{14}O_2$ in a closed chamber for 20 min., and grown for periods of 1 to 24 hr. in a normal atmosphere before harvesting. Synthesis of lignin is most rapid four to six hours from activation, the syringyl residues apparently being formed more slowly than the guaiacyl. A slower rate of formation persists for the remainder of a one-day period, probably as a result of recycling in the carbon pool. Cellulose acquires carbon-14 more rapidly than lignin during the period immediately following administration of $C^{14}O_2$, but after three or four hours when synthesis of lignin has become rapid, the total carbon-14 content of both components reaches about the same value.

INTRODUCTION

Earlier studies carried out in this laboratory (5) have traced the incorporation of labelled carbon dioxide into those portions of the wheat straw lignin molecule which yield phenolic aldehydes on nitrobenzene oxidation. The results showed that the total carbon-14 content of the syringaldehyde residue remained substantially constant after the first day, whereas that of *p*-hydroxybenzaldehyde exhibited a gradual decrease. That of the vanillin, after a small initial decline (possibly due to a decrease in more easily oxidizable guaiacyl-type lignans), also reached a constant level. No investigation was made, however, of the rate of incorporation of the C^{14} into lignin during the first day of activation. This paper reports the extension of the previous work to cover this early period.

The principles employed in these experiments were essentially those of the earlier studies, but several refinements in technique were developed. The use of a smaller activation chamber together with hydroponic cultivation of the wheat increased the convenience of activating the plants, and purification of the phenolic aldehydes was facilitated by precipitation as the 2,4-dinitrophenylhydrazones.

Preliminary experiments indicated that when plants were exposed to $C^{14}O_2$ for periods up to 20 min., and then permitted to continue growing in a normal atmosphere for further periods up to about an hour, very little carbon-14 entered the lignin. Considerable amounts appeared, however, after five hours of growth. A series of experiments was therefore carried out in which a group of similar plants were grown for periods of one to seven hours following activation. For comparison, other plants were grown for 24 hr. to obtain the approximate value of the maximum total activity.

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EXPERIMENTAL

Cultivation of the Wheat Plants

The Thatcher wheat plants used in these experiments were grown in aqueous nutrient solution. In addition to providing a reproducible source of inorganic nutrients, this procedure facilitated the use of the comparatively small activation chamber described below. The seeds were planted in moist Vermiculite, and the seedlings were allowed to grow to a height of about 10 cm. The roots were washed free of adhering particles, and placed in opaque jars in 500 ml. of nutrient of the following composition: $CaSO_4$, 136 mgm.; $Ca(NO_3)_2$, 164 mgm.; KNO_3, 101 mgm.; $MgSO_4$, 240 mgm.; KH_2PO_4, 136 mgm., *or* trace element solution, 0.1 ml.; demineralized water, 1 liter.

The composition of the trace element solution[3] was: H_3BO_3, 28 gm.; $ZnSO_4$, 2 gm.; $FeSO_4$, 50 gm.; $MnSO_4$, 20 gm.; $CuSO_4$, 2 gm.; concentrated H_2SO_4, 5 ml.; demineralized water, 1 liter.

The seedlings were allowed to grow through holes in the rubber stoppers of the jars, and were supported by cotton wool loosely stuffed in the holes, and later also by rings made from aluminum welding rod. A slow stream of air was passed continuously through the solution, which was changed twice weekly. All plants were grown in a greenhouse.

Activation with $C^{14}O_2$

The plants used for these experiments were 62 days old and well headed out; this age was selected because the rate of lignification is comparatively rapid during this period of growth (7). Before exposure to $C^{14}O_2$ they were kept in darkness for 24 hr.

The activation chamber was constructed of a 2 liter flask with 24/40 and 45/50 joints, and a 6 × 70 cm. vertical glass tube with a 45/50 joint, open at the top. The smaller joint of the flask held a T-tube, one end of which was closed with rubber tubing and a clamp, and the other connected to a 500 ml. siphon bottle. A small side arm just above the center of the large glass tube provided for admission of carbon dioxide from a small generating flask.

Each plant, with its supporting stopper, was removed from the nutrient and placed in the vertical tube. The tube was connected to the flask, the latter containing water into which the roots extended. The open end of the tube was tightly stoppered, and the air-tight assembly was placed in bright sunlight so that the rate of photosynthesis and incorporation of $C^{14}O_2$ would be at a maximum. A partial vacuum was created within by siphoning off as much water as would flow from the closed system. Then 0.2 ml. of 0.03 *M* $Na_2C^{14}O_3$, containing 20 μc. of carbon-14, was introduced by means of a syringe and needle through a serum bottle cap on the generating flask, which already contained 72% perchloric acid. Air was then sucked through a side arm in the carbon dioxide generator to sweep any remaining carbon dioxide into the chamber, and the process was repeated using 0.5 ml. of inactive 0.3% sodium carbonate solution. After 20 min. exposure the air in the chamber was swept out into a gas absorption

[3] *Phosphate and trace elements were added to alternate changes of nutrient; addition of both together resulted in iron deficiency symptoms, presumably due to the complexing of that element by phosphate* (2).

tower containing sodium hydroxide solution. After five minutes' vigorous sweeping the apparatus was disassembled and the plants replaced in nutrient, where they were allowed to grow in a normal atmosphere for periods of 1 to 24 hr.

Isolation of Lignin Degradation Products

Each plant, with heads and roots removed, was cut into 1 cm. lengths. The tissue was then killed rapidly by dropping it into water at about 90° in a Waring blendor and disintegrating for 10 min. The resulting mixture was transferred to a flask, and after a further 10 min. the supernatant was decanted off. The coarse fibrous material which remained was filtered off, sucked free of water, and dried at 105°. It was then extracted with ethanol–benzene, 1:2, in a Soxhlet apparatus until the solvent was colorless, dried thoroughly *in vacuo*, and ground to pass a 60 mesh screen.

Nitrobenzene oxidation of this material and separation of the resulting phenolic aldehydes was done by methods similar to those used in earlier work (6). One and a half milliliters of nitrobenzene and 25 ml. of 2 *N* sodium hydroxide were used per gram of dry plant material, and the 0.5 to 2 gm. samples were oxidized in a hydrogenator bomb. The residue of unattacked cellulose was filtered off on a glass frit, washed with water, and reserved for purification (see below). The filtrate and washings, after separation of the small oil layer, were made up to a convenient known volume (50–100 ml.) and 0.2 ml. was analyzed for phenolic aldehydes (6). To remove some impurities the remainder was extracted overnight with benzene in a continuous liquid–liquid extractor. Then, after acidification to pH 3, the phenolic aldehydes were extracted into benzene in the same way. The benzene was removed *in vacuo* and replaced by ethanol, and the ethanolic solution after concentration to about 1 ml. was chromatographed on three to five sheets of Whatman No. 1 paper, 46 × 57 cm. Ethanol solutions of the aldehydes separated in this way were decolorized with charcoal, concentrated to a volume of 1–2 ml., and treated with an excess of 2,4-dinitrophenylhydrazine reagent.[4] The precipitate of aldehyde 2,4-dinitrophenylhydrazone was filtered off, washed with cold ethanol, and recrystallized once from ethanol–pyridine. Further recrystallization of the active compounds produced no change in specific activity.

As there is some doubt that *p*-hydroxybenzaldehyde originates from lignin in wheat (5, 7) no measurements of its activity were made during this experiment.

Isolation of Cellulose

The residue of crude cellulose obtained following the nitrobenzene oxidation was further washed on the frit with ethanol and benzene, to remove adhering nitrobenzene and dissolved impurities. After drying, it was submitted to the series of treatments described by Lewis (3). One such series was usually sufficient to yield a pure white, fluffy cellulose.

To obtain a measure of the cellulose content of the original dry cell wall material, each sample was analyzed by Tettamanzi's modification of the Kürschner-Hoffer method (8). Samples of 10 to 30 mgm. were refluxed for one

[4] *This was prepared as described by Shriner and Fuson (4, p. 97) except that no water was added.*

hour with 5 ml. of concentrated nitric acid and methanol, in a 1:2 ratio by volume, and the residue of cellulose was filtered off and weighed.

Determination of Radioactivity

All samples were counted in the gas flow counting chamber of a Marconi type 138-660 proportional scaler.

The 2,4-dinitrophenylhydrazones were plated from pyridine solution on aluminum disks for counting, and the observed counts were corrected to "infinite thinness" from a self-absorption curve. The cellulose samples were burned in a combustion train, the carbon dioxide was absorbed in a carbonate-free sodium hydroxide solution, and precipitated as barium carbonate. This was plated for counting by filtration on disks of Whatman No. 3 filter paper, the counts being made at "infinite thickness".

In both cases the radioactivity was calculated as microcuries by reference to a carbon-14 standard obtained from the National Bureau of Standards, Washington. This standard, a solution of sodium carbonate, decayed at the rate of 1280 disintegrations per sec. per ml.; and after appropriate dilution it was precipitated and plated as barium carbonate, or plated at "infinite thinness" as sodium carbonate, using a procedure as nearly as possible the same as that used for the unknown samples.

RESULTS AND DISCUSSION

The changes in the carbon-14 content of vanillin and syringaldehyde during the first 24 hr. after activation of the plants are depicted in Fig. 1. It is evident that radioactive carbon begins to reach the lignin in appreciable quantities only after a lapse of several hours. From about four to five hours in the case of vanillin, and five to six hours in that of syringaldehyde the incorporation of carbon-14 is very rapid, and beyond this time there are indications of a levelling off of the curve. Despite this, however, the net gain of activity during the remainder of the 24 hr. period is about equal to that attained in the first seven hours, so it is evident that a slower incorporation persists until a maximum is reached, presumably near 24 hr. after activation. Whereas during the period of rapid synthesis the total carbon-14 content of the syringaldehyde seems always to lag behind that of the vanillin, this situation may be reversed in the period represented by the broken lines; in one experiment syringaldehyde of appreciably higher total activity was obtained after 24 hr. Such divergent results must represent fluctuations in relative rates of synthesis, and are not unexpected in view of variations among plants, and of the fact that different ones were, of course, used for each experiment. While the necessity for an additional methylation (probably by transmethylation from the methionine system (1)) would satisfactorily account for the early lag in the synthesis of the syringyl residue, direct comparisons with the guaiacyl should be made only with caution, as consistent differences in yield of vanillin and syringaldehyde during the oxidation could conceivably account for part of the difference.

It appears reasonable to conclude that most of the incorporation of carbon dioxide into lignin by the most direct synthetic route occurs during the first seven hours following activation. The slower build-up which continues for

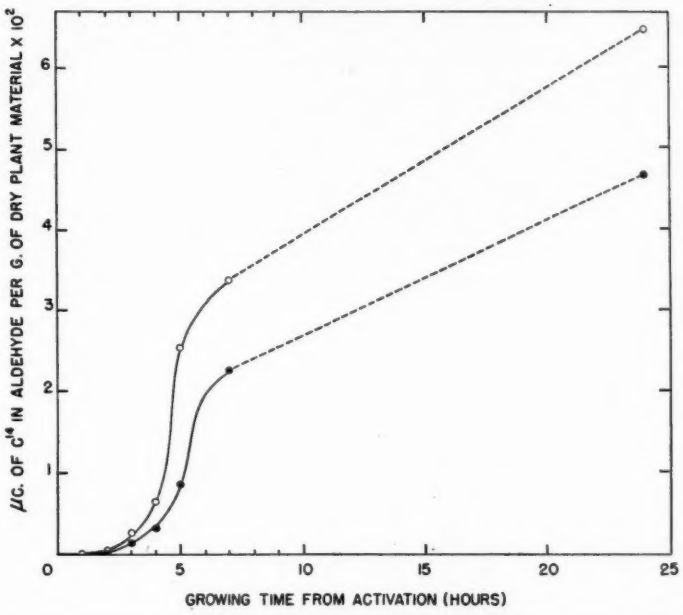

FIG. 1. Incorporation of C^{14} from carbon dioxide into vanillin and syringaldehyde obtained on lignin oxidation. The ordinate values are obtained by multiplying microcuries per millimole of the aldehyde (calculated from the count) by the millimoles of aldehyde per gram of ash-free straw (calculated from assay of the oxidation mixture). ○-Vanillin. ●-Syringaldehyde.

approximately 17 hr. more can be accounted for by the recycling of simple precursors in the plant's carbon pool, a process which would continue until they were respired, or converted into relatively stable compounds such as other cell wall constituents.

Because lignin is a substance of unknown chemical structure, it is not possible to obtain an accurate figure for the radioactive carbon content of the entire lignin fraction, as opposed to that of the derived phenolic aldehydes. In an earlier paper (7) however, a procedure was given for calculating total lignin based on the aldehyde yield from young plants and the 72% sulphuric acid lignin content of mature plants; this method is considered to give the closest approach to the true lignin content. As calculated in this way, the percentage of lignin is equal to the percentage of vanillin at the same age multiplied by a factor which at an age of 62 days equals 6. Consequently, if the total carbon-14 content of vanillin, as shown in Fig. 1, be multiplied by 6, it is possible to arrive at a value for the total carbon-14 content of lignin. The results are shown in the fourth column of Table I. The assumption is made, of course, that lignin is uniformly labelled (5), and it must be emphasized that these figures can be taken to represent only approximate values, as the data used to calculate the above factor were obtained with plants grown under somewhat different conditions.

Table I shows that, except during very early stages of synthesis, the total

TABLE I

DISTRIBUTION OF C^{14} IN CELL WALL AFTER ADMINISTRATION OF $C^{14}O_2$

Growing time from activation (hours)	μc. of C^{14} in cell wall derivative per gram of dry cell wall material				
	Vanillin	Syringaldehyde	Lignin (calc'd from vanillin)	Cellulose	Lignin / Cellulose
1	6.7×10^{-5}		4.0×10^{-4}	8×10^{-3}	0.05
2	4.1×10^{-4}	1.0×10^{-4}	2.5×10^{-3}	1.8×10^{-2}	0.14
4	6.40×10^{-3}	3.11×10^{-3}	3.8×10^{-2}	4.5×10^{-2}	0.85
5	2.55×10^{-2}	8.52×10^{-3}	1.5×10^{-1}	1.52×10^{-1}	1.0
7	3.38×10^{-2}	2.26×10^{-2}	2.0×10^{-1}	2.28×10^{-1}	0.9
24	6.48×10^{-2}	4.67×10^{-2}	3.9×10^{-1}	2.68×10^{-1}	1.5

carbon-14 entering lignin is roughly the same as that appearing in cellulose. The synthetic route to cellulose is probably the more direct, and it is to be anticipated that the discrepancy in favor of cellulose would be large in the early stages, and would be overcome as the rate of lignin formation from the administered C^{14} increased. It is of interest that even at the stage of maturity investigated, when the rate of lignification is greatest, the amount of carbon dioxide converted to lignin does not exceed that employed in the synthesis of cellulose.

It may also be noted that after 24 hr. the carbon-14 appearing both in lignin and in cellulose is of the order of $1\frac{1}{2}$ to 2% of that administered. The percentage of that actually absorbed by the plant is undoubtedly much higher, but the method of killing did not permit the total C^{14}-content to be determined with accuracy.

Further studies of possible intermediates between carbon dioxide and lignin are in progress.

ACKNOWLEDGMENT

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REARRANGEMENT IN THE REACTION BETWEEN 2-PHENYLETHYLAMINE-1-C^{14} AND NITROUS ACID[1]

By C. C. Lee and J. W. T. Spinks

ABSTRACT

On treatment with sodium nitrite, 2-phenylethylamine-1-C^{14} in aqueous hydrochloric, perchloric, acetic, or glacial acetic acid gives rise to products in which the C^{14} atoms have been rearranged from the C-1 to the C-2 position to an extent of 20.5 to 22.5%. The results are discussed on the basis of carbonium ions as possible reaction intermediates.

INTRODUCTION

The concept of a carbonium ion as an intermediate in the reaction between a primary amine and nitrous acid was elaborated in 1932 by Whitmore and Langlois (12). It is the basis for the presently accepted mechanistic interpretation of the Demjanow reaction for ring expansion (1). Products arising from rearrangements characteristic of carbonium ion processes have been observed for reactions of primary amines with nitrous acid; for example, the treatment of 2,2-diphenylethylamine with nitrous acid gave benzylphenylcarbinol (3, 6). The application of the tracer technique has recently furnished data, on rearrangements, which were previously unobtainable. Thus insight into the nature of the intermediate formed in interconversion reactions of cyclobutyl, cyclopropylcarbinyl, and allylcarbinyl derivatives was obtained from a study of the reaction of C^{14}-labeled cyclopropylcarbinylamine with nitrous acid (8). The migration ratios, with reference to phenyl, of several substituted aryl groups were determined by the nitrous acid induced rearrangement of the corresponding 2,2-diarylethylamine-1-C^{14} (3), and the formation of 1.5% of rearranged product, ethanol-2-C^{14}, was found when ethylamine-1-C^{14} was treated with nitrous acid (9).

This paper reports observations on the reaction between 2-phenylethylamine-1-C^{14} and nitrous acid.

RESULTS AND DISCUSSION

Carboxyl-labeled phenylacetic acid, prepared by carbonation of benzylmagnesium chloride with $C^{14}O_2$, was converted to the amide which in turn was reduced with lithium aluminum hydride to give 2-phenylethylamine-1-C^{14}. On oxidation with alkaline potassium permanganate, inactive benzoic acid was produced from the labeled amine, indicating that all the C^{14}-activity was in carbon-1.

When the labeled 2-phenylethylamine was dissolved in 10% hydrochloric, 10% perchloric, or 50% acetic acid and treated with an aqueous solution of sodium nitrite, the product isolated was 2-phenylethanol. Attempts to isolate, by fractional distillation, any styrene that might have resulted from possible elimination reactions were not successful. Part of the 2-phenylethanol was

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Contribution from the Department of Chemistry, University of Saskatchewan, Saskatoon, Sask.

TABLE I

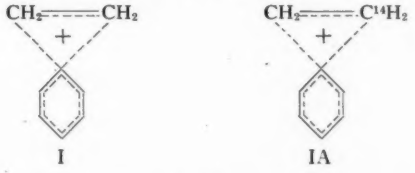
RADIOACTIVITY DATA FROM NITROUS ACID – 2-PHENYLETHYLAMINE-1-C^{14} REACTION

Compound	Reaction medium	Observed activity,[1] counts/min.		Corrected activity,[2] counts/min./mM.		% Rearrangement	
		Run I	Run II	Run I	Run II	Run I	Run II
$C_6H_5NHCSNHCH_2CH_2C_6H_5$[3]		1185 ± 11.2		303			
C_6H_5COOH[3]		0.5 ± 2.5		0.0			
$C_6H_5NHCOOCH_2CH_2C_6H_5$[4]	10% HCl	1240 ± 11.2	1232 ± 11.3	299	297		
C_6H_5COOH[4]	"	530 ± 5.4	502 ± 5.3	64.6	61.2	21.6	20.6
$C_6H_5NHCOOCH_2CH_2C_6H_5$[4]	10% $HClO_4$	1257 ± 11.3	1233 ± 11.2	303	297		
C_6H_5COOH[4]	"	552 ± 5.8	548 ± 5.8	67.3	66.8	22.2	22.5
$C_6H_5NHCOOCH_2CH_2C_6H_5$[4]	50% HOAc	1241 ± 11.5	1261 ± 11.5	299	304		
C_6H_5COOH[4]	"	515 ± 5.7	511 ± 5.7	62.8	62.3	21.0	20.5
$C_6H_5NHCOOCH_2CH_2C_6H_5$[4]	Glacial HOAc	1220 ± 11.3	1241 ± 11.4	294	299		
C_6H_5COOH[4]	"	531 ± 5.6	510 ± 5.5	64.7	62.2	22.0	20.8

[1] *"Infinitely thick" samples of constant geometry measured in windowless Q-gas counter.*
[2] *Observed activity × molecular weight × 10^{-3}.*
[3] *From 2-phenylethylamine-1-C^{14} before reaction with nitrous acid.*
[4] *From products of reaction between 2-phenylethylamine-1-C^{14} and nitrous acid.*

converted to its phenylurethan for activity determination. The remainder was oxidized with alkaline permanganate to benzoic acid. The radioactivity in the benzoic acid so obtained indicated a 20.5–22.5% rearrangement of the C^{14}-activity from the C-1 to the C-2 position during the conversion of the amine to the alcohol (Table I). When the reaction was carried out in glacial acetic acid and solid sodium nitrite was used, the product was 2-phenylethyl acetate which could be converted to 2-phenylethanol by treatment with lithium aluminum hydride or oxidized directly to benzoic acid with alkaline potassium permanganate. In the oxidation, probably the ester was first hydrolyzed by the alkaline medium. The degree of rearrangement in the acetate was the same as that obtained when the reaction was carried out in aqueous acids (Table I).

Recent interest in the structural representation of "nonclassical" carbonium ions poses the question whether the phenylethyl cation may have a bridged structure, the "ethylenephenonium ion" (I). In the reaction between 2-phenylethylamine-1-C^{14} and nitrous acid, the observed degree of rearrangement appears to have eliminated the possibility of the bridged ion as the only intermediate. If this reaction were to proceed solely and directly through IA, the resulting product, if isotopic effect were neglected, would consist of equal amounts of 2-phenyl-ethanol-1-C^{14} and 2-phenylethanol-2-C^{14}, equivalent to a 50% rearrangement.



I IA

To explain the observed results, one may assume that part of the reaction would lead to a rearranged product, arising, for example, from IA as reaction intermediate, and the remainder would proceed via some process or processes without rearrangement. One possible route that would lead to no rearrangement might be the formation of diazophenylethane in the course of the reaction:

$$C_6H_5CH_2C^{14}H_2\overset{+}{N}_2 \xrightarrow{-H^+} C_6H_5CH_2C^{14}HN_2 \xrightarrow{H_2O} C_6H_5CH_2C^{14}H_2OH + N_2.$$

However, Roberts and Yancey (9) have shown that in the ethylamine – nitrous acid reaction, less than 10% of the ethanol could have been formed via a diazohydrocarbon intermediate.

A more probable interpretation of the results is depicted below:

$$C_6H_5CH_2C^{14}H_2\overset{+}{N}_2 \xrightarrow{-N_2} C_6H_5CH_2C^{14}\overset{+}{H}_2 \rightleftarrows \text{IA} \rightleftarrows \overset{+}{C}H_2C^{14}H_2C_6H_5$$

IIA IIB

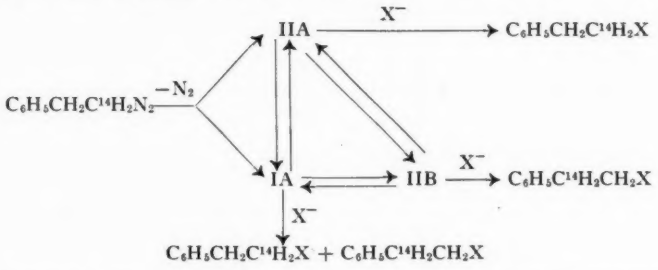
X = OH or OAc

$C_6H_5CH_2C^{14}H_2X$ $C_6H_5C^{14}H_2CH_2X$

The 2-phenylethyldiazonium ion may first give rise to IIA which can rearrange via IA to IIB. However, IIA may react with the conjugate base of the solvent

(OH^- or OAc^-) faster than it can rearrange, resulting in a predominance of products with no rearrangement as observed.

It should be pointed out that the existence of IA or IIB or both cannot be distinguished from radioactivity data alone. Of course, IA may react directly with OH^- or OAc^- to give the final products without proceeding through IIA and IIB. The possibility that IIA may rearrange directly to IIB, though unlikely, is not eliminated by the results from the tracer studies. Moreover, the observed degree of rearrangement can also be explained by assuming that the reaction proceeds partly through IIA and partly through the direct formation of IA. A series of reactions, analogous to that given by Roberts and Yancey (9), which includes all these possibilities is as follows:



It is not likely that these reactions would all take place during the treatment of 2-phenylethylamine with nitrous acid. All of them, however, are possibilities, appropriate combinations of which can satisfactorily account for the observed degree of rearrangement.

It is of interest to note that in the 3-phenyl-2-butanol system, Cram (4) found that with the tosylate of one of the stereoisomers, the ability of phenyl to participate in an intramolecular displacement reaction is about eight times the ability of hydrogen. The same trend is observed from a comparison of the degree of rearrangement reported here for 2-phenylethylamine-1-C^{14} and that reported by Roberts and Yancey (9) for ethylamine-1-C^{14}. The ratio of phenyl shift to hydride shift would be 20 to $22/1.5 \simeq 14$.

In view of the finding of Leete and co-workers (7) that an essentially 100% *p*-methoxyphenyl shift occurs in the conversion of *p*-vinylanisole to homoanisaldehyde by oxidation with yellow mercuric oxide and iodine, it would be logical to expand the present studies to include labeled substituted arylethylamines. Dr. J. D. Roberts has kindly informed one of us (C.C.L.) that studies with 2-*p*-nitrophenyl-, 2-*p*-methoxyphenyl-, as well as 2-phenylethylamine-1-C^{14} have been completed in his laboratory and that the results are in press.

EXPERIMENTAL

Phenylacetamide-1-C^{14}

Phenylacetic acid-carboxyl-C^{14} (400 mgm.), prepared according to the method given by Calvin *et al.* (2), and thionyl chloride (2 ml.) were heated on a steam bath under reflux for one-half hour. The excess thionyl chloride was removed *in vacuo*, and the residue taken up in dry ether. Ammonia, generated

by heating concentrated ammonium hydroxide, was bubbled into the ether solution. The amide, together with some ammonium chloride, precipitated. The product was collected by filtration, the inorganic salt being removed by thorough washing with distilled water. After drying, the colorless crystalline product weighed 288 mgm. (75%), m.p. 155–157°C. (lit. (2) m.p. 155–158°C.).

2-Phenylethylamine-1-C^{14}

In trial runs with nonlabeled materials, the reduction of phenylacetamide with lithium aluminum hydride according to the method of Uffer and Schlittler (11) required reaction times of 30–40 hr. for runs using 5–10 gm. of the amide. The yields obtained were of the order of 50%. By using a small amount of the amide in a fairly large volume of ether, thus overcoming the difficulties arising from the slight ether solubility of the amide, and isolating the product with the aid of added ordinary 2-phenylethylamine as carrier, the labeled amide was reduced to the amine as follows.

In a three-necked flask fitted with reflux condenser, dropping funnel, and stirrer was placed 400 mgm. of lithium aluminum hydride in 40 ml. of anhydrous ether. To the stirred solution, 250 mgm. of phenylacetamide-1-C^{14} (3.81×10^4 counts/min./mgm.,* total activity = 9.53×10^6 counts/min.) in 250 ml. of dry ether was added. Stirring was continued for six hours. The resulting mixture was decomposed by the addition of an excess of a 10% aqueous solution of sodium hydroxide. The ether was then separated and the aqueous layer extracted with more ether. To the combined ether solution, approximately 5 gm. of ordinary 2-phenylethylamine was added as carrier. After drying over anhydrous sodium sulphate, the ether was removed using an efficient column. The residue was distilled, the product boiling over the range of 195–198°C. (lit. (10) b.p. 198°C.). The recovery was 4840 mgm. (0.93×10^3 counts/min./mgm. of the hydrochloride,* total activity = 5.86×10^6 counts/min.). The yield, based on the total activity recovered, was 61.5%. A further quantity of inactive 2-phenylethylamine was added to give a stock of about 35 gm. of labeled amine which was used in all subsequent reactions.

N-Phenyl-N'-2-phenylethyl-1-C^{14}-thiourea

The phenylthiourea derivative of 2-phenylethylamine-1-C^{14} was prepared by treating the labeled amine with phenylisothiocyanate according to established methods (10). It melted at 135°C. (lit. (10) m.p. 135°C.). Its activity is recorded in Table I.

Oxidation of 2-Phenylethylamine-1-C^{14}

One gram of 2-phenylethylamine-1-C^{14}, 8 gm. of potassium permanganate, and 5 gm. of sodium hydroxide in 50 ml. of distilled water were placed in a 125-ml. erlenmeyer flask and heated on a steam bath for three hours with occasional shaking. The resulting mixture was cooled, the manganese dioxide removed on a suction filter and thoroughly washed with water. The filtrate and washings were acidified with sulphuric acid. Sodium bisulphite was added in small portions until the excess permanganate was reduced and the solution

**Measured as "infinitely thin" samples in a windowless counter.*

became colorless. The product was then extracted from the aqueous solution with ether. Removal of the solvent from the ethereal extract gave a colorless residue. Recrystallization from water gave 0.76 gm. (84%) of benzoic acid, m.p. 121°C. It was not radioactive (Table I) indicating that all the activity of the labeled amine was in the C-1 position.

Reaction between 2-Phenylethylamine-1-C^{14} and Nitrous Acid

A. *In Aqueous Acids*

In a 125-ml. erlenmeyer flask equipped with a magnetic stirrer was placed 3.63 gm. (0.03 mole) of 2-phenylethylamine-1-C^{14}. Ten per cent hydrochloric acid, 10% perchloric acid, or 50% acetic acid was added until all the amine was dissolved and the solution was acid to bromophenol blue. The flask was then immersed in an ice bath and agitated while a solution of 4.14 gm. (0.06 mole) of sodium nitrite in 10 ml. of water was added portionwise, more acid being introduced at the same time to keep the reaction medium acidic. The ice bath was then removed and the reaction mixture stirred at room temperature for one hour. During this period of stirring, an additional 2.07 gm. (0.03 mole) of solid sodium nitrite was added, together with more acid when necessary to maintain an acidic medium. The resulting mixture was finally heated on a steam bath for 10 min. before it was cooled and extracted with ether. The extract, after being washed with dilute acid, alkali, and water and being dried over anhydrous sodium sulphate, was concentrated until all the ether had been removed. The residue was distilled at atmospheric pressure in a distillation flask with a long side arm serving as air condenser. The product, 2-phenylethanol, boiled at 215–218°C. (uncorrected), n_D^{25} 1.5318; phenylurethan, m.p. 79–80°C. (lit. (5) $n_D^{16.8}$ 1.5337; phenylurethan, m.p. 79–80°C.). The yields from various runs are listed below. Trial distillations under reduced pressure showed little, if any, improvement in the yield.

Reaction medium	% Yield
10% HCl	61, 66
10% $HClO_4$	50, 54
50% HOAc	76, 78

B. *In Glacial Acetic Acid*

To a stirred solution of 3.63 gm. (0.03 mole) of 2-phenylethylamine-1-C^{14} in 30 ml. of glacial acetic acid was added portionwise 6.21 gm. (0.09 mole) of solid sodium nitrite. Stirring was continued for one-half hour. The mixture was then heated on the steam bath for an additional one-half hour. After cooling, the resulting material was poured into ice–water and extracted with ether, the extract being thoroughly washed with alkali and distilled water. Distillation of the residue, after removal of the ether by evaporation from the dried extract, gave 2-phenylethyl acetate, b.p. 218–220°C. (uncorrected), n_D^{25} 1.5064 (lit. (5) n_D 1.5108). The yields on duplicate runs were 2.96 gm. and 3.15 gm. (69% and 73%). The material did not react with phenylisocyanate.

An ether solution of 3.00 gm. of the acetate was added dropwise to 0.75 gm. of lithium aluminum hydride in 50 ml. of anhydrous ether. The mixture was stirred for two hours and then decomposed with dilute hydrochloric acid. The

product was worked up by ether extraction and distillation of the residue from the extract. The 2-phenylethanol recovered weighed 1.70 gm. (68%), n_D^{25} 1.5321. With phenylisocyanate, it gave the phenylurethan, m.p. 79–80°C., in 90% yield.

Phenylurethan of 2-Phenylethanol

Equal weights (0.5 to 1.0 gm. scale) of 2-phenylethanol and phenylisocyanate and a few drops of pyridine, placed in a 10 ml. erlenmeyer flask, were heated on the steam bath for 15 min. The resulting mixture was cooled in an ice bath. A few milliliters of petroleum ether, b.p. 30–60°C., was added and the flask rubbed with a stirring rod whereby the urethan readily crystallized. It was collected by filtration and thoroughly washed with petroleum ether, the yields being of the order of 90%. On recrystallization from chloroform – petroleum ether mixture, it melted at 79–80°C. (lit. (5) m.p. 79–80°C.). The radioactivity data are recorded in Table I.

Oxidation of 2-Phenylethanol or 2-Phenylethyl Acetate

The oxidation of the products of reaction between 2-phenylethylamine-1-C^{14} and nitrous acid in the various acid media used was carried out in alkaline potassium permanganate. The conditions were the same as given above for the oxidation of 2-phenylethylamine-1-C^{14}, the amine being replaced by the alcohol or the acetate. The yields of recrystallized benzoic acid ranged between 70 and 80%. Measurements of the activity of various samples are tabulated in Table I.

ACKNOWLEDGMENT

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NOTES

A NOTE ON THE PREPARATION OF CHLORINE GAS CONTAINING Cl^{36} [1]

By F. Brown, A. Gillies,[2] and W. H. Stevens

On several occasions in this laboratory, it has been important to obtain chlorine gas in practically quantitative yield from either potassium chloride or dilute hydrochloric acid containing Cl^{36}. The standard methods for obtaining chlorine gas from these compounds, for example, oxidation by $KMnO_4$, MnO_2, or $K_2Cr_2O_7$, in acid solutions, do not give high yields.

A method for obtaining a 95–100% yield of radioactive chlorine gas from $NaCl^{36}$ was recently reported by Woeber (1). The procedure consisted of adding cold fuming sulphuric acid to sodium chloride dissolved in ice-cold superoxol and sweeping the chlorine evolved out of the generator with an inert gas.

Previous to this, we had developed a somewhat similar method which we feel is more easily controlled and perhaps less hazardous when applied to larger scale preparations.

The method is as follows: Two to three equivalents of potassium peroxysulphate are added to either an approximately 0.5 *N* hydrochloric acid solution or an equivalent potassium chloride – sulphuric acid solution. An inert gas stream is gently bubbled through the mixture and the mixture is heated at 70–75°C. The production of chlorine gas is easily controlled by cooling or warming the reaction mixture. Practically no reaction occurs below 65°, and at 70–75° the reaction proceeds quite smoothly. If the chlorine is required dry, the gas stream may be passed through concentrated sulphuric acid. The chlorine gas may be frozen out of the carrier gas stream (e.g. argon, or helium), if desired, using a liquid nitrogen cooled trap. Some oxygen is also evolved during the reaction from decomposition of peroxysulphate by water, but below 75°C. this reaction is quite slow.

On a one millimole scale, the reaction requires 10 to 15 min. On a hundred millimole scale, the reaction requires one half to three quarters of an hour.

The yield of chlorine, determined in several inactive runs by absorbing the chlorine in potassium iodide solution and titrating the iodine liberated with thiosulphate, was 98–100%. Tracer experiments were also done to determine the completeness of the chloride oxidation. Solutions of hydrochloric acid containing Cl^{36} were counted using a dipping geiger tube. After reaction, the residual solutions were again counted. Only 1½–2% of the original radioactivity remained in the solution.

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[1] *Issued as A.E.C.L. No. 61.*
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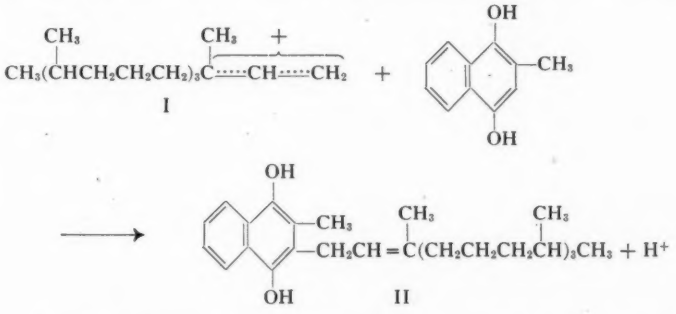
VITAMIN K_1 LABELED WITH C^{14}

By C. C. Lee, F. C. G. Hoskin, L. W. Trevoy, L. B. Jaques, and J. W. T. Spinks

Vitamin K_1, labeled with C^{14}, was synthesized using a method developed by Dr. Otto Isler of F. Hoffman-La Roche, A. G., Basle (4), with modifications suited to small-scale synthesis. It involves the reaction between isophytol (3,7,11,15-tetramethyl-1-hexadecen-3-ol) and 2-methyl-1,4-naphthohydroquinone with boron trifluoride as catalyst. Isophytol is known to isomerize readily in the presence of phosphorus tribromide and ligroin to give phytyl bromide (5, 8), and Dr. Isler (4) found that it can successfully replace phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) in Fieser's (2, 3) well-known vitamin K_1 synthesis. One may suggest that in the presence of the acid catalysts used, an identical cationoid intermediate, I, is derived from either phytol or isophytol, which then reacts with 2-methyl-1,4-naphthohydroquinone through an electrophilic displacement to give the hydroquinone of vitamin K_1 (II).



In the Hoffmann-La Roche process, the reaction product was hydrogenated using a special palladium on calcium carbonate catalyst which was found suitable for reducing vitamin K_1 to its hydroquinone (6). This treatment eliminates any loss of the hydroquinone product through oxidation, during the course of the reaction, to the liquid quinone (vitamin K_1 itself) which would be washed away during the isolation of the solid hydroquinone. After the hydrogenation, the isolated hydroquinone is taken up in ether and oxidized to vitamin K_1 with silver oxide.

In the radiochemical synthesis, the starting material was 2-methyl-C^{14}-1,4-naphthohydroquinone prepared from C^{14}-labeled vitamin K_3 (7) according to the method of Fieser (3). The activity of this starting material was 1.56×10^5 counts/min./mgm.* or 2.68×10^7 counts/min./mM. (approximately 40 μc./mM.). The resulting 2-methyl-C^{14}-3-phytyl-1,4-naphthoquinone (vitamin K_1) had an activity of 5.82×10^4 counts/min./mgm. or 2.62×10^7 counts/min./mM. With alcoholic potassium hydroxide, the labeled vitamin K_1 gave a positive Dam-Karrer color test (2). Its ultraviolet absorption

**Measured as infinitely thin samples in a windowless Q-gas counter.*

spectrum, measured in alcoholic solution using a Beckmann quartz spectrophotometer, was practically identical with that of an authentic sample and agreed with that recorded by Ewing and co-workers (1). The radiochemical purity was further demonstrated by its conversion to a solid derivative, the diacetate of 2-methyl-C^{14}-3-phytyl-1,4-naphthohydroquinone (2). Applying the principles of isotopic dilution, the diacetate, on two successive recrystallizations, accounted for 94.3% and 96.0%, respectively, of the vitamin K_1 activity used in the preparation of the derivative.

EXPERIMENTAL

A mixture of 500 mgm. of 2-methyl-C^{14}-1,4-naphthohydroquinone, 400 mgm. of isophytol, and 0.1 ml. of 45% boron trifluoride in ethyl ether (Eastman Organic Chemicals) was dissolved in 2 ml. of dry dioxane. The solution, under a nitrogen atmosphere in a small glass-stoppered flask, was mechanically shaken at room temperature for 24 hr. The resulting dark brown solution was taken up in 20 ml. of ether and washed first with 10 ml. of distilled water to remove the dioxane, then with three 10-ml. portions of 2% aqueous potassium hydroxide, containing 5% sodium hydrosulphite, to remove the unreacted 2-methyl-C^{14}-1,4-naphthohydroquinone. The yellow alkaline washings were combined and extracted once with ether. This extract was, in turn, washed with aqueous alkali containing sodium hydrosulphite before it was added to the original ether solution. After the ethereal solution was dried over anhydrous sodium sulphate, it was concentrated to a small volume for transfer to the flask used in the subsequent hydrogenation.

The reaction product was placed in a 15 ml. hydrogenation bottle, fitted with a magnetic stirrer, and was subjected to evacuation by the water pump to remove all the remaining ether. To the viscous residue, 3 to 4 ml. of petroleum ether and about 100 mgm. of palladium catalyst were added. The mixture was hydrogenated at room temperature under ordinary pressure for one-half hour. The hydroquinone of vitamin K_1 separated as a white voluminous precipitate. It was transferred under nitrogen into a 15 ml. centrifuge tube, with the aid of an additional few milliliters of cold petroleum ether, and centrifuged after thorough cooling in an ice–salt bath. The brown supernatant liquid was drawn off and the solid hydroquinone washed two or three times with a few milliliters of cold petroleum ether, the washings being removed by centrifugation. The final washing was only slightly colored. The washed product was dissolved in 10 ml. of dry ether, the catalyst suspended in the ether solution being easily removed by centrifugation.

To the ether solution of the hydroquinone, 500 mgm. of silver oxide was added and the mixture agitated by means of a magnetic stirrer for one-half hour. A small quantity of anhydrous sodium sulphate was then added as drying agent. The resulting ethereal solution of vitamin K_1 was separated from the oxidizing and drying agents by centrifugation. With the removal of the solvent, finally *in vacuo*, 146 mgm. of vitamin K_1 was obtained as a clear yellow oil. A small droplet dissolved in 95% alcohol gave, with alcoholic potassium hydroxide, an intense indigo blue color which changed with time

to purple and finally to dull red, a positive Dam-Karrer test. The ultraviolet absorption spectrum of an alcoholic solution of the product was practically identical with that of an authentic sample (Merck and Co.) (1). The maximum peak of absorption occurring at 249 mμ had an optical density of 0.800 for a concentration of 2 mgm. of vitamin K_1 in 100 ml. of 95% alcohol. The bulk of the labeled vitamin was dissolved in sesame oil, approximate concentration 10 mgm./ml., for administration to experimental animals.

From the alkaline washings of the original reaction mixture, 224 mgm. of unreacted 2-methyl-C^{14}-1,4-naphthohydroquinone was recovered. The 146 mgm. of labeled vitamin K_1 obtained thus represents a yield of 20% based on the used 2-methyl-C^{14}-1,4-naphthohydroquinone. This compares favorably with the reported yield of Fieser (3) which, based on the hydroquinone of vitamin K_3, would be of the order of 15%.

The unreacted 2-methyl-C^{14}-1,4-naphthohydroquinone was recovered through acidification of the alkaline washings with sulphuric acid followed by extraction with ether. The ethereal extract was shaken with 10% aqueous sodium hydrosulphite to keep the desired material in the reduced form, dried over anhydrous sodium sulphate, and evaporated to about one milliliter. Sulphur, which resulted from the decomposition of the hydrosulphite when the washings were acidified, separated out on cooling. It was filtered off and washed with a small volume of cold ether to leach out all the hydroquinone of vitamin K_3. On addition of petroleum ether to the sulphur-free ether solution, the unreacted 2-methyl-C^{14}-1,4-naphthohydroquinone separated as a grayish powder and was collected by filtration. Further purification was effected by dissolving the recovered material in ether, shaking with 10% hydrosulphite solution, drying, and again adding petroleum ether to the concentrated ethereal solution. The purified hydroquinone of vitamin K_3 thus recovered weighed 224 mgm. It was of sufficient purity for use in vitamin K_1 synthesis. When treated with silver oxide in ether, it reverts readily to vitamin K_3, isolated by sublimation, m.p. 103–104°C. (uncorrected) (lit. (7), m.p. 103–104°C.).

A weighed quantity (147.6 mgm.) of the sesame oil solution of labeled vitamin K_1, together with 497.6 mgm. of ordinary vitamin K_1 (Merck and Co.), was subjected to reductive acetylation with acetic anhydride and zinc dust in the presence of a small amount of pyridine according to the procedure given by Fieser (2). The colorless diacetate, crystallized from methanol, weighed 420 mgm. On recrystallization from ethanol, it melted at 59–60°C. (uncorrected) (lit. (2), m.p. 59–60°C.). Radioactivity measurements showed that the diacetate, after one and then two recrystallizations, accounted for 94.3% and 96.0%, respectively, of the vitamin K_1 activity used.

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We should like to express our sincere appreciation to Dr. Otto Isler for transmitting the details of his process, which was modified for this synthesis, and to Messrs. Hoffman-La Roche Inc., Nutley, N.J., for a generous supply

of isophytol and palladium catalyst. The financial assistance given by the National Research Council of Canada is also gratefully acknowledged.

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N-*BIS*-(β-N-ARYLCARBAMYLOXYETHYL)ARYLAMINES[1]

BY A. F. MCKAY and W. G. HATTON

Some new N-*bis*-(β-N-arylcarbamyloxyethyl)arylamines (II) have been prepared by the reaction of arylisocyanates with N-*bis*-(β-hydroxyethyl)arylamines (I). The properties of these compounds are given in Table I.

$$\underset{\text{I}}{R\text{–}N(CH_2CH_2OH)_2} + 2R'\text{–}N=C=O \rightarrow \underset{\text{II}}{R\text{–}N(CH_2CH_2OC(O)NHR')_2}$$

EXPERIMENTAL[2]

N-bis-(β-Hydroxyethyl)benzylamine

N-*bis*-(β-Hydroxyethyl)benzylamine (b.p.$_{0.5\text{ mm.}}$ 140–142°C.; $n_D^{24.8}$ 1.5355) was prepared in 95% yield by the addition of ethylene oxide to benzylamine. The procedure was similar to the previously described method (3) of preparation of *bis*-(β-hydroxyethyl) *n*-butylamine. Gabriel and Stelzner (2) report a boiling point of 225–225.5°C. at 40 mm. pressure for N-*bis*-(β-hydroxyethyl)benzylamine.

N-bis-(β-Hydroxyethyl) p-Anisidine

Fifty grams (0.406 mole) of *p*-anisidine were dissolved in 100 cc. of methanol and brought to reflux. During a period of eight hours, 39.4 gm. (0.895 mole) of ethylene oxide were introduced under the surface of the amine solution. After removal of solvent and excess ethylene oxide, 84.5 gm. (99.2%) of crystalline N-*bis*-(β-hydroxyethyl) *p*-anisidine (m.p. 67.5–70°C.) was obtained. This compound was purified by trituration with petroleum ether and filtration. A final melting point of 72.5°C. was obtained. N-*bis*-(β-Hydroxyethyl) *p*-anisidine prepared from ethylene chlorohydrin and *p*-anisidine has been described (1, 4) as melting at 73°C. A mixed melting point determination between samples prepared by both methods gave no depression.

Preparation of di-N-Arylcarbamyl Derivatives of N-bis-(β-Hydroxyethyl)arylamines

Two mole equivalents of the arylisocyanate are added to one mole equivalent

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[2] *All melting points are uncorrected. Microanalyses by C. W. Beazley, Skokie, Illinois.*

of N-*bis*-(β-hydroxyethyl)arylamine in dry benzene. After addition is complete, the benzene solution may be warmed to bring the reaction to completion. In this manner the reaction products were prepared in quantitative yields. Purification was effected by crystallizing from 95% ethanol. The compounds obtained by this procedure are described below in Table I.

TABLE I

$RN(CH_2CH_2OC(O)NHR')_2$

R	R′	M.p., °C.	C		H		N		Cl	
			Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found
p-Anisyl	*p*-Chlorophenyl	144.5–145	57.91	58.28	4.86	5.10	8.11	8.25	13.67	13.80
p-Anisyl	*p*-Tolyl	140–140.5	67.92	67.57	6.54	6.48	8.80	9.10		
p-Anisyl	Phenyl	102–102.5	66.82	67.15	6.06	6.16	9.35	9.44		
p-Anisyl	Benzyl	86–86.5	67.92	67.77	6.54	6.41	8.80	8.95		
Benzyl	*p*-Chlorophenyl	112–112.2	59.76	59.45	5.02	5.21	8.37	8.24	14.11	14.36

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